

OXYGEN SUPPLY TO IMMOBILIZED CELLS

Patrick Adlercreutz



Department of Biotechnology

Lund 1985

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To Kaisa and my parents

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LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals.

- I Adlercreutz P, Holst O and Mattiasson B (1985)
Characterization of Gluconobacter oxydans immobilized in calcium alginate.
Appl Microbiol Biotechnol 22:1-7
- II Adlercreutz P and Mattiasson B (1982)
Oxygen supply to immobilized cells: 1. Oxygen production by immobilized Chlorella pyrenoidosa.
Enzyme Microb Technol 4:332-336
- III Adlercreutz P, Holst O and Mattiasson B (1982)
Oxygen supply to immobilized cells: 2. Studies on a coimmobilized algae-bacteria preparation with in situ oxygen generation.
Enzyme Microb Technol 4:395-400
- IV Adlercreutz P and Mattiasson B (1982)
Oxygen supply to immobilized cells. 3. Oxygen supply by hemoglobin or emulsions of perfluorochemicals.
Eur J Appl Microbiol Biotechnol 16:165-170
- V Adlercreutz P and Mattiasson B (1984)
Oxygen supply to immobilized cells. 4. Use of p-benzoquinone as an oxygen substitute.
Appl Microbiol Biotechnol 20:296-302
- VI Adlercreutz P (in press)
Oxygen supply to immobilized cells. 5. Theoretical calculations and experimental data for the oxidation of glycerol by immobilized Gluconobacter oxydans cells with oxygen or p-benzoquinone as the electron acceptor.
Biotechnol Bioeng

1. INTRODUCTION

Organisms can be divided into three groups depending on their need for oxygen. One, strict anaerobes that cannot use oxygen at all and often cannot tolerate it, two, facultative anaerobes that can live either with or without oxygen and three, aerobes that need oxygen. This final group includes many bacteria and fungi as well as all the higher animals.

In aerobic cells oxygen has two main functions. One is to serve as the terminal electron acceptor in the respiratory chain and the other to serve as a substrate in reactions catalyzed by oxygenases and oxidases. Quantitatively, the terminal electron acceptor function is the most important. When oxygen accepts electrons from the respiratory chain, water is formed. The respiratory activity of cells varies considerably. Small, actively metabolizing cells, such as many bacteria, have a high respiratory activity.

In unicellular organisms the oxygen needed can diffuse through the cell wall and reach the sites in the cell where it is needed. Higher animals have circulation systems to provide their cells with the oxygen, nutrients and other substances needed. Another function of the circulation system is to transport excretion products from the cells. In humans, about half the blood volume consists of red blood cells whose main function is to transport oxygen and carbon dioxide.

Cells contain a vast variety of enzymes capable of catalyzing different reactions. Some of these reactions can be exploited industrially for the production of different substances. When aerobic cells are used in industrial processes oxygen supply is often critical (Enfors and Mattiasson 1983). If the cells do not receive enough oxygen productivity will be low. In industry, it is often advantageous to use high cell densities in reactors so that a high volumetric productivity is achieved but of course an increase in the cell density will cause an increase in oxygen demand. When immobilized cells are used in biotechnical processes, high cell densities are often used. Thus the productivity of immobilized preparations of aerobic cells is often limited by the supply of oxygen.

Sometimes it is advantageous to use purified enzymes as catalysts instead of whole cells. When oxygen demanding enzymes are used, oxygen supply is often a limiting factor just as for aerobic cells.

This thesis will treat oxygen supply to heterogenous catalysts such as immobilized cells. Different methods of increasing the oxygen supply to immobilized cells are presented. These methods can also be used to supply oxygen to other oxygen demanding systems.

1.1. Solubility of oxygen in water

Compared to other liquids water is a poor solvent of oxygen (see section 4.3.1.). As most bioconversions are performed in water, the low solubility of oxygen in the reactant solution is a severe problem in oxygen demanding processes.

At a constant temperature the solubility of oxygen in water, $c(O_2)$, is proportional to the partial pressure of oxygen in the gas phase, $p(O_2)$, according to Henry's law:

$$c(O_2) = \frac{p(O_2)}{H} \quad (1)$$

where H is a constant.

The solubility of oxygen in water decreases with increasing temperature. Tables of the solubility of oxygen in water are given by e.g. Montgomery et al. (1964) and Atkinson and Mavituna (1983)(p.734).

Additions of soluble substances in water normally decrease the solubility of oxygen. The effects of different components of fermentation media on gas solubilities were reviewed by Schumpe et al. (1982).

1.2. Determination of oxygen concentrations

The concentration of dissolved oxygen in a solution can be determined in many different ways. Some methods measure the concentration directly while other methods measure the oxygen pressure of the solution, which is defined as the partial pressure of oxygen in the gas phase in equilibrium with the solution.

One method which is often used to measure dissolved oxygen concentrations is the Winkler method (American Society for Testing and Materials 1980). With this method the oxygen of the sample oxidizes Mn(OH)_2 to MnO(OH) which is then dissolved in acid. The Mn(III) ions then oxidize I^- to I_2 , which is titrated with thiosulfate.

As part of the present project, a method for measuring oxygen concentrations with an enzyme thermistor was developed (Adlercreutz and Mattiasson 1982). Glucose oxidase and catalase were immobilized on a solid support and packed in a small column. Buffer was pumped continuously through the column. Samples containing glucose and oxygen were injected. The heat evolved in the enzymatic reactions was measured with a sensitive thermistor. With an excess of glucose present, the device was used to measure oxygen concentrations. This method was well suited for measurements of oxygen concentrations in solutions containing oxygen carriers. Many oxygen carriers, such as hemoglobin, interfere with the Winkler method.

For continuous measurement of the oxygen pressure in water solutions, oxygen electrodes are convenient. Both polarographic and galvanic oxygen electrodes are commercially available. A short review of oxygen electrodes is given by Atkinson and Mavituna (1983)(p.740). In papers I,II and VI a polarographic electrode was used to measure oxygen pressures. In order to convert the oxygen pressure data to oxygen concentrations, the electrode must be calibrated using a method that actually measures the oxygen concentration, e.g. the Winkler method.

2. MODEL SYSTEM

As the aim of the present work was to study different methods to increase the oxygen supply to immobilized biocatalysts, a model system with a high oxygen demand and severe diffusion limitations was chosen. The model system consisted of Gluconobacter oxydans (ATCC 621) cells immobilized in calcium alginate gel. This system will be described in some detail below.

2.1. Acetic acid bacteria

Acetic acid bacteria are well-known because of their ability to perform incomplete oxidations of organic compounds in the presence of oxygen (Asai 1968). Some of the products formed are of commercial interest.

Acetic acid bacteria belong to the family Acetobacteraceae (De Ley et al. 1984). The family consists of two genera, Acetobacter and Gluconobacter. In papers I and III-VI Gluconobacter oxydans (ATCC 621) was used as an example of an oxygen demanding organism. The reaction studied was the oxidation of glycerol to dihydroxyacetone (DHA) (Fig. 1).



Fig. 1. Oxidation of glycerol to dihydroxyacetone by Gluconobacter oxydans.

DHA is currently produced by fermentation. At present, the main use of DHA is as an artificial suntanning agent for the cosmetic industry. Other applications are possible, especially if DHA can be produced at a low cost. In this study the reaction was used simply as a model reaction. It was not the aim to develop a process for the production of DHA.

Many of the incomplete oxidation reactions of acetic acid bacteria can be described by Bertrand-Hudson's rule. This rule states that only polyols having the configuration in Fig. 2 are oxidized to the corresponding ketoses. The simplest example is the oxidation of glycerol to DHA (Fig. 1).

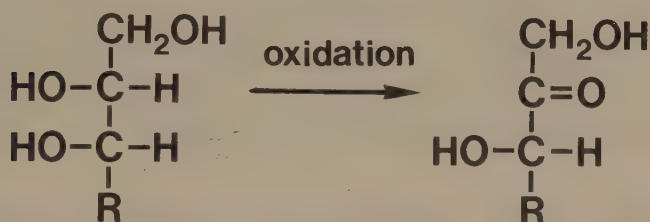


Fig. 2. Oxidation according to Bertrand-Hudson's rule.

The enzymes responsible for the incomplete oxidation reactions of acetic acid bacteria are dehydrogenases which are often membrane bound. Some of these have been isolated and characterized (Table 1).

Enzyme	organism	coenzyme	references
alcohol DH	<u>G.suboxydans</u> (IFO 12528)	PQQ	Adachi et al. 1978 Ameyama et al. 1981a
aldehyde DH	<u>G.suboxydans</u> (IFO 12528)	PQQ	Adachi et al. 1980 Ameyama et al. 1981a
D-glucose DH	<u>G.suboxydans</u> (IFO 12528)	PQQ	Ameyama et al. 1981b
D-fructose DH	<u>G.industrius</u> (IFO 3260)	N.S.	Ameyama et al. 1981c
2-keto-D-			
gluconate DH	<u>G.melanogenus</u> (IFO 3293)	Fla	Shinagawa et al. 1981
D-sorbitol DH	<u>G.suboxydans</u> (IFO 3254)	Fla	Shinagawa et al. 1982
D-gluconate DH	<u>G.dioxyaceticus</u> (IFO 3271)	Fla	Shinagawa et al. 1984

Table 1. Examples of membrane bound dehydrogenases which have been isolated from Gluconobacter strains and characterized. (DH= dehydrogenase, PQQ= pyrrolo quinoline quinone, Fla= flavin, N.S.= not stated).

The enzyme of interest in this investigation, the glycerol dehydrogenase of G. oxydans (ATCC 621), might be identical with the D-sorbitol dehydrogenase from the same strain described by Baker and Claus (1983). This enzyme was not isolated but its properties were shown to be different from those of the D-sorbitol dehydrogenase characterized by Shinagawa et al. (1982). Both the D-sorbitol dehydrogenase and the glycerol dehydrogenase activities of G. oxydans (ATCC 621) were found in the membrane fraction of homogenized cells. They were both solubilized with β -octylglucoside but destroyed by Triton X-100 (Baker and Claus 1983, Adlercreutz unpublished).

The coenzyme of the glycerol dehydrogenase is probably a flavin or pyrrolo quinoline quinone (PQQ). PQQ is a recently discovered coenzyme which is found in several bacterial dehydrogenases (Salisbury et al. 1979, Duine et al. 1979, Ameyama et al. 1981a)(Table 1).

The dehydrogenases are not directly reoxidized by oxygen, but they are linked to respiratory chains containing cytochromes. Some of the cytochromes are tightly bound to dehydrogenases. Oxygen is normally the terminal electron acceptor in the respiratory chains.

2.2 Immobilization

Immobilized biocatalysts are enzymes, cells or organelles (or combinations of these) which are in a state that permits their reuse (European Federation of Biotechnology 1983). Six different types of immobilization methods can be distinguished: adsorption, affinity (biospecific) immobilization, covalent coupling (including cross-linking), entrapment in a three-dimensional polymer network, confinement in a liquid-liquid emulsion and finally capture behind semi-permeable membranes (Mattiasson 1983b). These methods have been reviewed several times (Mattiasson 1983b, Kennedy and Cabral 1983, Nilsson 1984). Here only certain details concerning immobilization in alginate gels will be given as this was the method used in papers I-VI.

Immobilization renders the biocatalyst easier to handle. An immobilized biocatalyst can easily be recovered from the reaction products. Sometimes immobilization improves the stability of the biocatalyst. Other advantages of immobilization are that high biocatalyst concentrations can be used and that continuous processes can easily be developed. The main disadvantage of immobilization, with the exception of the extra costs for immobilization itself, is that mass transfer of substrates to the catalyst and of products from the catalyst is retarded. In some cases, the increased mass transfer resistance is difficult to overcome and the productivity is then decreased.

2.2.1. Immobilization in alginate gels

Alginate is a polysaccharide isolated from marine brown algae. It is a linear copolymer of D-mannuronic acid and L-guluronic acid (Fig. 3) (Smidsröd et al. 1972). Alginates from different algae or different parts of the plants can vary in monomer composition, chain length and arrangement of the monomers. The properties of alginates are therefore quite variable. Sodium and potassium alginates are soluble in water while the salts of most divalent and multivalent cations are insoluble and form strong gels. Alginates with a high proportion of guluronic acid are the best gel formers.

Klein et al. (1983) measured the pore size of different alginate gels by steric exclusion chromatography. Dextrans were used as standards. The exclusion limit was in the range 40 000 - 110 000 in molecular weight which corresponds to hydrodynamic radii of between 5 and 8 nm.

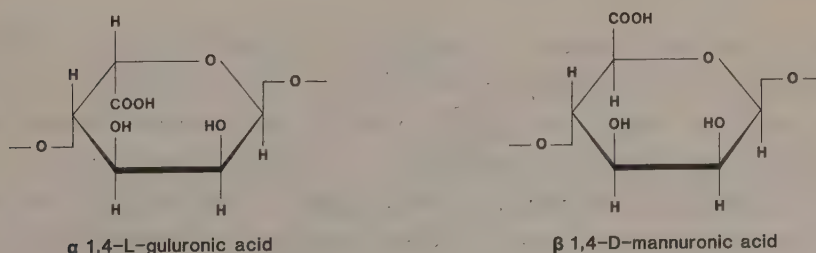


Fig. 3. The monosaccharides of alginate.

Insoluble alginate gels have been widely used to immobilize cells. Hackel et al. (1975) studied phenol degradation by Candida tropicalis cells immobilized in aluminium alginate gel and other matrices. Kierstan and Bucke (1977) immobilized microbial cells, subcellular organelles and enzymes in calcium alginate gels.

In practice, cell immobilization in calcium alginate starts with the dispersion of the cells in a solution of alginate, usually sodium alginate. This mixture is brought into contact with a solution containing calcium ions. A gel forms immediately. Often the cell alginate mixture is dropped into a calcium chloride solution so that spherical beads are formed. Immobilization in alginate gel is a mild immobilization technique which can also be used for sensitive cells. Other immobilization methods often involve harmful chemicals or elevated temperatures.

The calcium alginate gel is stable as long as the calcium ions remain in the gel. Solutions containing ions that bind calcium ions (e.g. citrate) or form precipitates with calcium ions (e.g. phosphate) should be avoided during the operation otherwise the gel may dissolve. Often the reactant solution is supplemented with calcium ions to stabilize the gel. Calcium alginate gels can be stabilized so that they are also stable in solutions that normally dissolve the gels (Birnbaum et al. 1981). Some of the methods used are treatment with polycations (Veliky and Williams 1981), replacement of calcium with barium ions (Paul and Vignais 1980) and crosslinking with glutaraldehyde or tannins (Takata et al. 1977).

2.3. Characterization of the model system

In paper I the model system, G. oxydans cells immobilized in calcium alginate gel, was characterized according to the guidelines of the Working Party on Immobilized Biocatalysts of the European Federation of Biotechnology (European Federation of Biotechnology 1983). The reaction studied was the oxidation of glycerol to DHA (Fig. 1). The pH optimum of the reaction was 5.0 and the temperature optimum was 40°C. However, the operational stability was better at 30°C. The glycerol concentration required to obtain half the maximal reaction rate was

about 5 mM for both immobilized and free cells. Studies of the influence of particle size, cell density and oxygen concentration on the reaction rate revealed that the supply of oxygen was a rate limiting step. At low concentrations of glycerol and high concentrations of DHA a slight product inhibition was noted. No loss of activity in the immobilized preparation was observed after storage for 68 days at +4°C. Investigation of the operational stability revealed a half-life of 5 days in a buffered glycerol solution. However, if nutrients are added, a far better operational stability of the system can be achieved (Seiskari et al. 1985). In this case the cells were actively growing during the operation.

3. KINETICS AND MASS TRANSFER IN IMMOBILIZED BIOCATALYSTS

The reaction rate of a chemical process is determined by mass transfer effects and kinetic effects. Under some conditions, mass transfer limits the overall reaction rate while under other conditions the kinetics are rate limiting. The situation is principally the same for traditional chemical processes and biotechnical processes. This text will deal with oxygen demanding reactions carried out with immobilized biocatalysts. In this case oxygen supply often limits the overall reaction rate.

Oxygen transfer from the gas phase to the cells in the immobilized cell preparation can be divided into six steps (Enfors and Mattiasson 1983) (Fig. 4). Which step or steps are important for the overall reaction rate depends on the properties of the immobilized cell preparation and on the conditions during the reaction. Sometimes all the steps take place in the same reactor, but some steps may also take place outside the main reactor. When immobilized cells are used in packed bed reactors oxygen transfer from the gas phase to the liquid phase normally takes place in an oxygenator separate from the packed bed reactor.

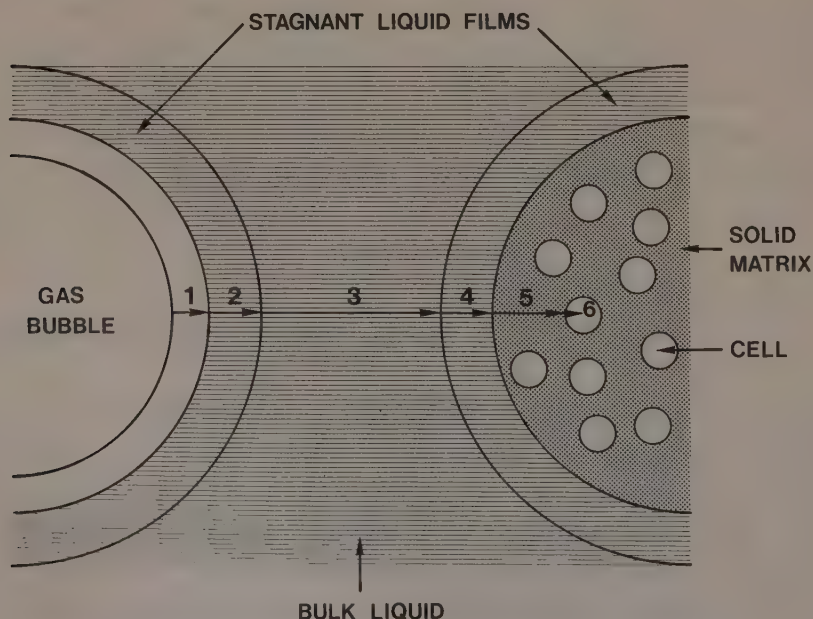


Fig. 4. Schematic representation of the different steps in oxygen transfer to immobilized cell preparations. For detailed explanations see text.

3.1. Gas-liquid oxygen transfer

Steps 1-3 in Fig. 4 are similar in both immobilized cell processes and in conventional fermentation processes and will be treated only briefly in this text. Much work has been carried out concerning oxygen supply to fermentation processes (Atkinson and Mavituna 1983). Often gas-liquid oxygen transfer is a rate limiting step. The mass transfer resistance in the gas film (step 1) can normally be neglected compared to the mass transfer resistance in the liquid film (step 2) (Atkinson and Mavituna 1983, Calderbank and Moo-Young 1961).

3.2. External and internal oxygen transfer

The main part of this chapter will concern steps 4 and 5 in Fig. 4. These two steps are of special importance in the use of immobilized biocatalysts. Step 4 represents diffusion through the stagnant liquid film surrounding the solid particles and will be called 'external mass transfer' below. Diffusion through the matrix to the cells (step 5) will be called 'internal mass transfer'. Step 6 in Fig. 4 represents transport through the cell wall to the oxygen consuming sites of the cells. In processes using free cells, step 5 is omitted and step 4 may represent the mass transfer resistance in the stagnant liquid film surrounding the cells. This mass transfer resistance is normally negligible compared to the gas-liquid mass transfer resistance.

In many immobilized biocatalysts internal mass transfer resistance is larger than the external mass transfer resistance. However, it has often been observed that the reaction rate when using an immobilized biocatalyst is influenced by the stirring speed in the reactor. This is an indication that external mass transfer is not negligible.

As a result of the oxygen consumption inside the immobilized biocatalyst and the external and internal mass transfer resistances, the oxygen concentration inside the matrix will be lower than in the bulk liquid phase. The oxygen concentration profiles can be calculated. In Fig. 5 one concentration profile for a catalyst with a very low oxygen consuming activity and one for an immobilized preparation with highly active cells are shown.

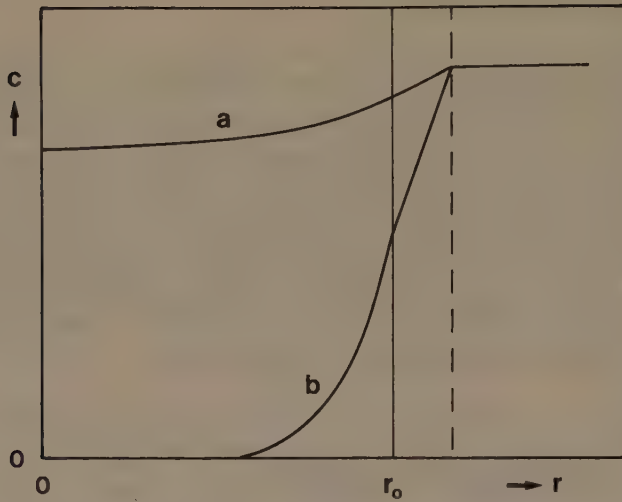


Fig. 5. Oxygen concentration profiles in spherical particles with the radius r_0 . Curve 'a' represents an immobilized biocatalyst with a low oxygen consuming activity and curve 'b' one with a high activity. The broken line represents the end of the stagnant liquid film around the particles.

The concentration profiles of oxygen in biocatalyst preparations have been measured directly. Kasche and Kuhlmann (1980) carried out direct measurements in the unstirred diffusion layer outside immobilized biocatalysts using an oxygen microelectrode. The results were in agreement with predictions by theoretical analysis. The thickness of the diffusion layer was not negligible even at the highest relative velocity used. Moreover, concentration profiles in mycelial pellets (Wittler et al. 1984) and microbial slime (Bungay et al. 1969) have been measured.

3.2.1. Diffusion of oxygen in gels

The mass transfer mechanism for both external and internal mass transfer is diffusion. Therefore, the diffusion of oxygen will be examined in some detail.

Many different methods have been used to measure the diffusivity of oxygen in water. Tham et al. (1970) reviewed the results obtained by nine different methods. The values varied between 1.90 and $2.55 \cdot 10^{-9} \text{ m}^2/\text{s}$ at 25°C .

The diffusion of molecules in gels is often slower than the diffusion in free solution. Some experimental determinations of internal (also called effective or apparent) diffusivities of oxygen in polysaccharide gels have been published. The internal diffusivity of oxygen in 2% agar gel at 30°C was estimated to be 70% of that of oxygen in water (Sato and Toda 1983). The measurements were made in an apparatus especially designed for diffusion experiments. The diffusivity was little affected by the presence of inactivated cells up to a concentration of $8 \text{ kg dry weight}/\text{m}^3$.

In addition to these direct measurements, the internal diffusivities of oxygen in different gels have been determined by indirect methods. Hiemstra et al. (1983) calculated the internal diffusivity of oxygen in barium alginate gels containing Hansenula polymorpha cells from measurements of the oxygen consumption rate of the immobilized preparations. They found that the internal diffusivity decreased with increasing cell density. At cell densities between 3 and $15 \text{ kg dry weight of cells}/\text{m}^3$ it was 25% of the diffusivity in water.

In paper VI the internal diffusivity of oxygen in calcium alginate gel was calculated from measurements of the oxygen consumption rate of the immobilized cell preparations. No clear dependence of the internal diffusivity on the cell density ($\leq 48 \text{ kg dry weight of cells}/\text{m}^3$) was observed. The internal diffusivity in the gel was 77% of that in water.

Some different mathematical expressions concerning the relationship between the diffusivity in solution, D , the internal diffusivity, D_{is} , and the porosity of the catalyst, θ , have been presented. Cheetham (1983) claimed that D_{is}/D is proportional to θ . In other expressions D_{is}/D is said to be proportional to $\theta^{1.5}$ or θ^2

(Boersma et al. 1979). Klein and Washausen (1979) presented a different equation:

$$\frac{D_{is}}{D} = (1 - X)^2 \cdot e^{-4V_p} \quad (2)$$

where X is the cell density (g wet weight/cm³) and V_p the volume fraction of the polymer. This equation shows a strong influence of the cell density on the internal diffusivity.

In conclusion, there is some controversy concerning the diffusivity in gel materials. Probably both the polymer and the cells of an immobilized cell catalyst influence the internal diffusivity of substrates to some extent. More experimental data for well defined systems are needed to deduce the proper equations.

3.3. Mathematical models of immobilized biocatalysts

The reaction rate obtained with an immobilized biocatalyst depends on many different parameters. Some of these are characteristics of the enzyme chosen while others depend on how immobilization is done or on the conditions during bioconversion.

Both external and internal mass transfer have been subject to extensive theoretical work. Models of varying complexity have been developed to describe mass transfer and reaction kinetics of catalyst preparations, both traditional chemical catalysts and biocatalysts. Here the model used in paper VI will be presented.

In this model the biocatalyst was immobilized in spherical gel beads. The beads were assumed to have the same radii and the biocatalyst was assumed to be uniformly distributed in the beads. Other assumptions in the model were that the system was at steady state and that the presence of reaction products did not affect the reaction rate. The partition coefficient for the substrate between the liquid and the solid phase was assumed to be 1.

For the free biocatalyst, it was assumed that the mass transfer resistances could be neglected and that Michaelis-Menten kinetics were applicable:

$$v_{\text{free}} = k_2 \cdot \rho \cdot \frac{c_{\text{bs}}}{c_{\text{bs}} + K} \quad (3)$$

where v_{free} is the reaction rate, ρ is the biocatalyst concentration (kg dry weight/m³), c_{bs} is the bulk substrate concentration while k_2 and K are the kinetic constants. Intrinsic kinetic constants refer to free biocatalyst and inherent kinetic constants refer to the kinetic behaviour of the immobilized biocatalyst in the absence of diffusional limitations (Cheetham 1980). The product $k_2 \cdot \rho$ is often called v_{max} .

For the immobilized biocatalyst the reaction rate was normally limited by the mass transfer of the substrate. The local reaction rate, v_{loc} , obtained in a small section of a bead, was determined by the local substrate concentration, c_{is} :

$$v_{\text{loc}} = k_2 \cdot \rho \cdot \frac{c_{\text{is}}}{c_{\text{is}} + K} \quad (4)$$

The local substrate concentration varies with the distance to the surface of the bead. As c_{is} is less than c_{bs} , the observed reaction rate, v_{obs} , is lower than the rate obtained with free biocatalyst, v_{free} . The observed reaction rate can be expressed in terms of diffusion of substrate into the catalyst particles:

$$v_{\text{obs}} = -D_{\text{is}} \cdot \left(\frac{dc_{\text{is}}}{dr} \right)_{r=r_0} \cdot \frac{3}{r_0} \quad (5)$$

where D_{is} is the internal diffusivity of the substrate in the gel, r is the distance from the center of the bead and r_0 is the bead radius.

The overall effectiveness factor, η_0 , is a measure of the influence of diffusion limitations on the reaction rate. In the chemical engineering literature the effectiveness factor is defined as the ratio of the actual reaction rate to the rate that would be obtained in the absence of diffusion resistance. When immobilized biocatalysts are used, the

effectiveness factor is often expressed as the ratio of the reaction rate of the immobilized biocatalyst to the rate obtained with the same amount of free biocatalyst (Kasche 1983, Hiemstra et al. 1983, paper VI):

$$\eta_0 = \frac{v_{\text{obs}}}{v_{\text{free}}} \quad (6)$$

The two definitions are equivalent if the rate obtained with the free biocatalyst is the same as that of the immobilized biocatalyst in the absence of diffusion limitations. The intrinsic kinetic constants are then equal to the inherent kinetic constants. Differences between intrinsic and inherent kinetic constants may be due to alterations of the biocatalyst during immobilization or partition effects caused by the matrix.

In the stagnant liquid film around the catalyst particles mass transfer occurs via diffusion. The following equation is applicable:

$$k_c \cdot (c_{bs} - c_{os}) = - D_{is} \cdot \left(\frac{dc_{is}}{dr} \right)_{r=r_0} \quad (7)$$

where k_c is the mass transfer coefficient for the external mass transfer and c_{os} is the substrate concentration at the particle surface.

Internal mass transfer occurs via diffusion. The diffusion in the matrix has been treated in section 3.2.1. The equations for internal mass transfer and reaction kinetics can be combined. The following equation is applicable to spherical particles:

$$D_{is} \cdot \left(\frac{d^2 c_{is}}{dr^2} + \frac{2}{r} \cdot \frac{dc_{is}}{dr} \right) = \frac{k_2 \cdot \rho \cdot c_{is}}{K + c_{is}} \quad (8)$$

The boundary conditions which apply are:

$$\begin{aligned} r = 0 \quad \frac{dc_{is}}{dr} &= 0 \\ r = r_0 \quad c_{is} &= c_{os} \end{aligned} \quad (9)$$

Equations such as (8) cannot be solved analytically. Several numerical methods have been used to solve the equation. In paper VI the method of Fink et al. (1973) was used. External as well as internal mass transfer was considered. From the calculations both the substrate concentration profiles in the catalyst particles and the overall reaction rate were deduced.

Models similar to the one described above have been developed for many types of catalysts. Catalysts with particle shapes other than spheres have also been treated. For cylindrical particles and flat membranes exact equations can be found while irregular particles are often approximated as spheres and the equations above are used. Models for enzymes encapsulated in membranes have been developed (Fink et al. 1973). Factors that can be included in the models are inhibitions of different kinds such as substrate inhibition (Yamané et al. 1981). If cell growth occurs in the catalyst the model must be extended (Sato and Toda 1983).

Sometimes the solubility of the substrate is different in the solid matrix compared to the liquid phase. One example is the use of hydrophobic gels which can be favourable for the transformation of hydrophobic substrates. Theoretical calculations concerning the kinetics of immobilized enzymes when such partition effects must be taken into account were presented by Yamané et al. (1981).

3.4. Practical use of calculations on immobilized biocatalysts

With theoretical calculations it is possible to obtain an overview of the different factors governing the overall reaction rate. Such data are useful in the planning of industrial processes.

Data for immobilized biocatalysts can be presented in many different ways. Often, the reaction rate or the overall effectiveness factor, η_0 , is given as a function of some parameter. In paper VI, η_0 was often expressed as a function of the bulk substrate concentration, c_{bs} . Fig. 6 shows experimental data and theoretical curves calculated using the model described in section 3.3. The overall effectiveness factor can also be expressed as a function of the cell density in the particles (Fig. 7) or some other parameter.

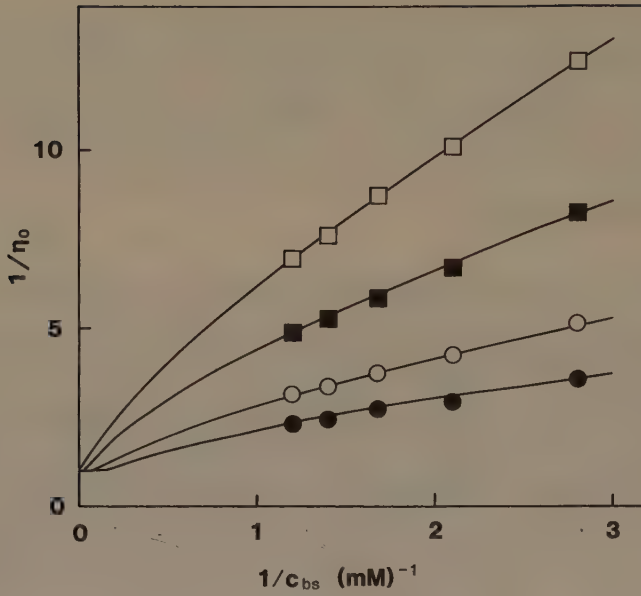


Fig. 6. Theoretical calculations (solid lines) and experimental data (marked points) for the oxidation of glycerol by immobilized *G. oxydans* cells with oxygen as the electron acceptor. The particle radii were 1.1-1.2 mm. The following cell densities were used: 8.9 kg/m³ (●), 16.3 kg/m³ (○), 29.2 kg/m³ (■) and 48.1 kg/m³ (□)(from paper VI).

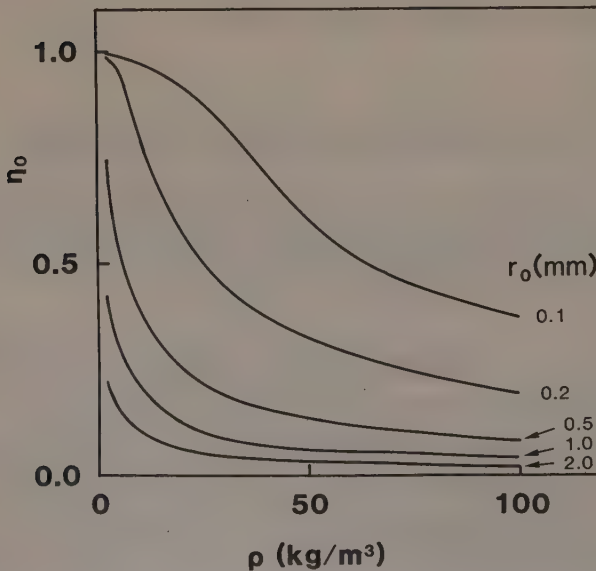


Fig. 7. Theoretical calculations of the overall effectiveness factor as a function of the cell density for different particle radii (from paper VI).

Many authors have combined certain important parameters to a Thiele modulus. The traditional Thiele modulus for a biocatalyst is defined as follows:

$$\Phi = L \cdot \left(\frac{v_{\max}}{K \cdot D_{is}} \right)^{0.5} \quad (10)$$

where $L = r_0/3$ for spherical particles. However, not even when Thiele moduli are used are single curves obtained in the diagrams. As the bulk substrate concentration is not included in the modulus a range of curves for different bulk substrate concentrations is obtained.

3.4.1. Determination of kinetic constants for immobilized biocatalysts

One motive for performing theoretical calculations along with experiments on immobilized biocatalysts is that the inherent kinetic constants can then be deduced. One method of doing this was presented by Engasser and Horvath (1973). To determine the $v_{\max}$ a preparation with only a small mass transfer resistance must be prepared. This is a limitation of the method. When moderate mass transfer resistances are at hand, K and D_{is} can also be calculated using the Engasser and Horvath method. Alternative methods to determine the inherent kinetic constants from measurements on immobilized enzyme systems were presented by Hamilton et al. (1974), who also considered external mass transfer, and by Kobayashi and Laidler (1973).

Often researchers want to characterize the kinetic behaviour of their immobilized biocatalysts in a simple way. So-called apparent kinetic constants are sometimes used for this purpose. When free enzymes or cells are used, data from kinetic experiments are often plotted with $1/v$ as a function of $1/c_{bs}$ (Lineweaver-Burk plot) or with v as a function of v/c_{bs} (Eadie-Hofstee plot). These plots give straight lines and the $v_{\max}$ and K can be deduced from the slopes and intercepts. When data for immobilized biocatalysts having high mass transfer resistances are plotted as Lineweaver-Burk plots, curved lines are obtained. A review concerning the kinetics of immobilized enzymes has been written by Goldstein (1976). Several authors have presented results from theoretical calculations concerning immobilized

enzymes where mass transfer has influenced the reaction rate. Regan et al. (1974) showed their results as Lineweaver-Burk plots. Deviations from the straight lines were apparent in most cases. Engasser and Horvath (1973) performed similar calculations. They also presented the equations for the curved lines in Lineweaver-Burk plots.

In limited ranges the Lineweaver-Burk plots of immobilized biocatalysts approach straight lines and sometimes these lines are extrapolated to give apparent K and $v_{\max}$ values. These parameters often have little physical significance (Hamilton et al. 1974, Goldstein 1976, Radovich 1985) but are often used because it is difficult to find better ways of expressing the kinetics of immobilized biocatalysts more simply.

3.5. Experimental studies of oxygen demanding reactions carried out using immobilized biocatalysts

Some oxygen consuming immobilized biocatalysts have been thoroughly characterized. Oxygen transfer in an immobilized enzyme preparation was studied by Tsukamoto et al. (1982). Glucose oxidase and catalase were immobilized on a carbon support. The experimental results were compared to theoretical calculations using a model based on Michaelis-Menten kinetics and internal diffusion. External mass transfer was not considered. The apparent kinetic constants were determined. While $v_{\max}$ was considerably lower in the immobilized preparation compared to the soluble enzymes, K was little affected by the immobilization. The internal diffusivity of oxygen was also calculated.

Sato and Toda (1983) studied the oxygen uptake rate of immobilized growing Candida lipolytica cells. The cells were immobilized in a 2% agar gel. The oxygen consumption rate was compared to the results of theoretical calculations based on Michaelis-Menten kinetics and internal oxygen diffusion. External oxygen transfer was not considered. K and $v_{\max}$ were determined from measurements using homogenized biocatalyst. The correlation between experiments and theory was rather good.

The oxygen consumption of Hansenula polymorpha cells immobilized in barium alginate gel was studied by Hiemstra et al. (1983). These cells oxidize methanol. The mathematical model used was based on Michaelis-Menten kinetics and internal oxygen transfer. External oxygen transfer was not considered. K and $v_{\max}$ were determined using free cells. The internal diffusivity of oxygen in the gels was determined. The scatter of experimental data was considerable.

The oxidation of glucose by Gluconobacter oxydans immobilized in calcium alginate was studied by Tramper et al. (1983). In this study the influence of the alginate type and the alginate concentration was investigated as well as the influence of the particle size. It was apparent that the oxygen transfer limited the reaction rate and that high alginate concentrations caused low reaction rates.

Nitrosomonas europaea cells immobilized in calcium alginate were studied by van Ginke1 et al. (1983). The reaction catalyzed by these cells was the oxidation of ammonium ions:



It was shown that the overall reaction rate was often limited by the mass transfer of the substrates and products. When a reactant solution with a low buffering capacity was used, accumulation of hydrogen ions in the gel particles caused a decrease in the activity of the cells. Furthermore diffusion of the substrates, oxygen and ammonium ions, greatly influenced the reaction rate. The dependence of the reaction rate on the oxygen concentration was expressed as apparent K and $v_{\max}$ values.

The results obtained with N. europaea were compared to data obtained with a mixed population of nitrifying bacteria immobilized in calcium alginate (Tramper et al. 1985). As expected the reproducibility of the results was lower with the mixed culture when compared to the pure strain. The general characteristics of the two preparations were largely similar except for the operational stability which was poorer in the case of the mixed culture. The kinetic constants for oxygen consumption by the immobilized mixed culture were expressed both as apparent and intrinsic constants. The apparent and intrinsic $v_{\max}$ values were approximately equal but the intrinsic K values were smaller than the apparent K values.

In paper VI the experimental results obtained with G. oxydans cells immobilized in calcium alginate were compared to theoretical data. The mathematical model used involved Michaelis-Menten kinetics and external as well as internal mass transfer. The reaction studied was the oxidation of glycerol to DHA with oxygen or p-benzoquinone as the electron acceptor. In both cases the correlation between the calculations and the experimental data was good. Higher production rates were obtained using p-benzoquinone as the electron acceptor than when oxygen was used. The reasons for this were that p-benzoquinone gave a higher $v_{\max}$ value and its solubility was higher than for oxygen.

4. METHODS OF INCREASING THE OXYGEN SUPPLY TO IMMOBILIZED BIOCATALYSTS

The different methods used to increase the oxygen supply to immobilized biocatalysts can be divided into nonchemical and chemical methods. The nonchemical methods aim to decrease the oxygen transfer resistances in the reactor or to increase the driving force for oxygen transfer. No extra chemicals are used. Among the chemical methods some involve in situ oxygen generation while others make use of oxygen carriers. This text will mainly treat the chemical methods. The rate of oxygen demanding reactions can also be increased by the use of artificial electron acceptors. This method will also be treated here although it is not strictly a method of increasing the oxygen supply.

4.1. Nonchemical methods

Much work has been carried out in order to develop fermentors suitable for aerobic fermentations. Normally gas-liquid oxygen transfer is the rate limiting step so the aim must be to decrease this oxygen transfer resistance. Many different aeration systems have been used to achieve good oxygen transfer. As a rule stirring must be vigorous to create small gas bubbles and to promote effective oxygen transfer. However, vigorous stirring is often not suitable when immobilized cells are used because the matrix may deteriorate. One reactor design which has been used successfully with immobilized cells is the airlift fermentor where the gas bubbles provide oxygen and also agitate the reaction medium. The oxygen transfer characteristics of airlift fermentors were recently reviewed by Bello et al. (1985).

The oxygen transfer resistances that are of special importance to immobilized cell preparations are steps 4 and 5 in Fig. 4. In order to minimize the external mass transfer resistance (step 4), the relative velocity between the catalyst particle and the liquid phase should be high. This is achieved by vigorous stirring in a tank reactor and a high flow rate in a packed bed reactor. A pulsed flow can be used instead of a high flow rate (Ghommidh et al. 1982).

Internal oxygen transfer (step 5) is often the main oxygen transfer resistance in immobilized cell preparations. Several methods can be

used to decrease this limitation. One way is to decrease the size of the catalyst particles. This is sometimes the best method of decreasing oxygen transfer limitations. However, very small particles are difficult to handle. The diffusion limitations are also decreased if the cell density is decreased, but then more catalyst and often larger reactors must be used. A good alternative is to immobilize cells just on the surface of the catalyst particles. However, this method also entails a decrease in the catalytic capacity per unit volume of the catalyst so that more catalyst must be used. The properties of the matrix used for immobilization will also influence the mass transfer resistance. It is advantageous to use a highly porous matrix to facilitate internal diffusion.

One way of decreasing the oxygen transfer resistances is to increase the partial pressure of oxygen in the gas phase used to oxygenate the liquid in the reactor. Makhotkina et al. (1981) observed an increased productivity when air was replaced by oxygen-enriched air in the production of DHA from glycerol with G. oxydans cells immobilized in polyacrylamide. Ghommidh et al. (1982) found that acetic acid production by immobilized Acetobacter cells was increased by more than a factor of 2 when air was replaced by oxygen. In papers I and VI the rate of DHA production of immobilized G. oxydans cells was shown to be greatly influenced by the oxygen concentration in the bulk liquid phase.

Another way of increasing the partial pressure of oxygen in the gas phase is to increase the total gas pressure. Sato et al. (1981) showed that the oxygen absorption rate in a fermentor, measured by the sulfite method, increased proportionally to the 0.4-0.8th power of the total pressure. However, the use of pressures above atmospheric pressure puts extra demands on the reactor.

4.2. In situ oxygen generation

One way of increasing the oxygen supply to a process is to generate oxygen in the reactor. Some of the different methods of achieving this are photolysis of water by algae, decomposition of hydrogen peroxide and electrolysis of water.

4.2.1. Photolysis of water by algae

During photosynthesis algae and cyanobacteria produce oxygen. This has been used to increase the supply of oxygen to immobilized cell preparations.

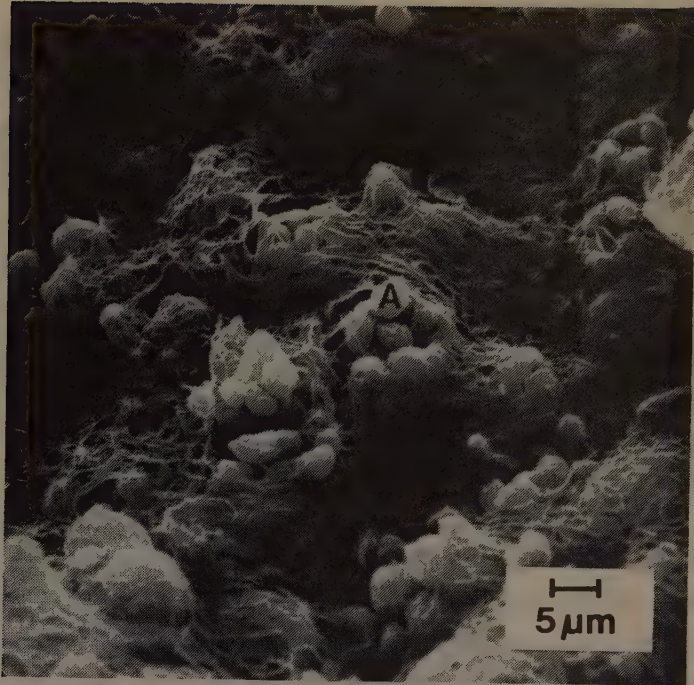
In paper II the oxygen production of Chlorella pyrenoidosa cells immobilized in calcium alginate gel was studied. Additions of carbonate greatly increased the oxygen production rate. Moreover the addition of nitrate also increased the oxygen production rate but was not as effective as carbonate. The optimal pH value for oxygen production was 7.0 when carbonate was used as the substrate. Light intensity greatly influenced the rate of oxygen production. When carbonate was used as the substrate the oxygen production rate did not reach the maximum in the range tested (≤ 33.5 klx). The immobilized algae produced oxygen continuously for 30 days in a column reactor. During this time the algae multiplied and no decrease in oxygen production was noted.

In paper III the algae were co-immobilized with G. oxydans cells. The oxygen produced by the algae was effectively used by the bacteria. Carbonate was used as the substrate for the algae and glycerol for the bacteria. The production of DHA by the bacteria was measured. The optimal conditions for the co-immobilized preparation were pH 6.0, a carbonate concentration of 30 mM and a light intensity of 20.5 klx. The co-immobilized preparation was quite stable. No significant decrease in activity was noted during the 6 days of operation.

The co-immobilized algae-bacteria preparations were studied with sweep electron microscopy (Fig. 8). From the photographs obtained it was evident that the algae multiplied in the preparations. After some days of operation small groups of algae, probably originating from single cells, were seen in the gel particles (Fig. 8a).

Co-immobilization of algae and bacteria was also performed by Wikström et al. (1982) who immobilized Chlorella vulgaris cells together with Procidencia cells. They noted an increase in the production of keto-acid from amino acid due to increased oxygen supply in the co-immobilized preparation as compared to a preparation without algae.

a



b

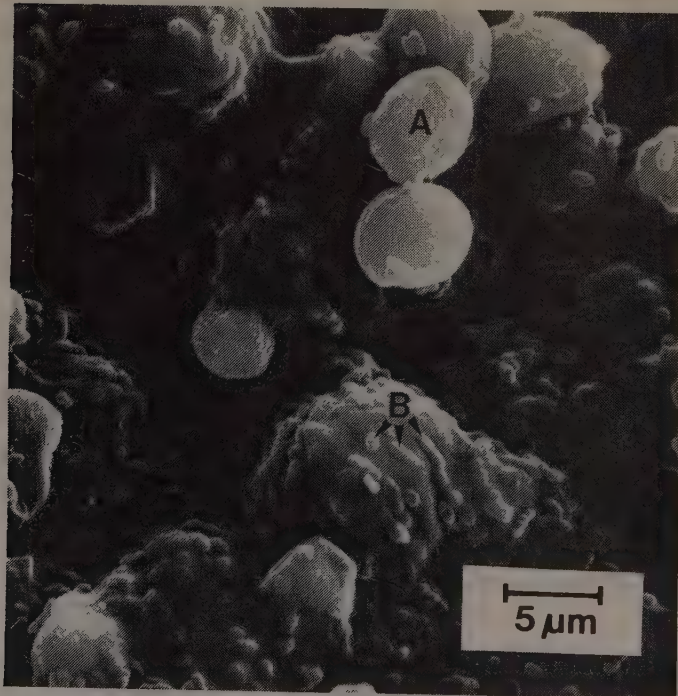
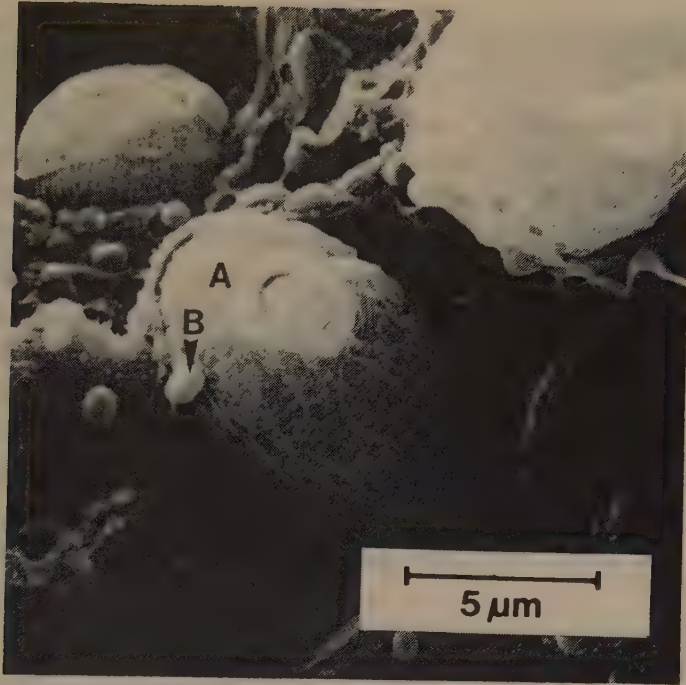
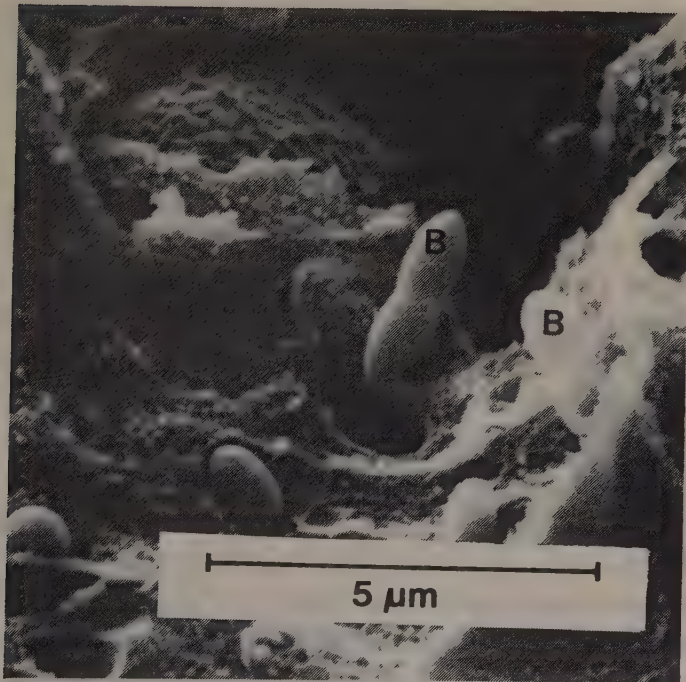


Fig. 8. Sweep electron micrographs of a co-immobilized algae-bacteria preparation in calcium alginate. After a few days of operation the alginate beads were freeze-fractured and fixed with glutar aldehyde and OsO_4 . A = algal cells (*Chlorella pyrenoidosa*), B = bacterial cells (*Gluconobacter oxydans*).

c



d



4.2.1.1. Reactor design

For DHA production in paper III, light was a limiting factor. The reactor design used (packed bed reactor) is not suitable for the practical application of light dependent processes, although it was suitable for studies of the influence of different parameters on the production rate. For practical applications a reactor with dispersed biocatalyst particles is better. Some alternatives concerning the reactor design are the loop reactor of Lee and Pirt (1981) and the flume reactor of Laws et al. (1983). Different light sources can be used. Sunlight is the cheapest alternative. However, electric illumination is better if the process has to be controlled. Combinations of sunlight and electric light might be a good compromise.

4.2.1.2. Advantages and limitations of co-immobilization

Immobilized biocatalysts have mainly been used to carry out onestep reactions. This is so, of course, with biocatalysts containing just one enzyme, but even in immobilized cell preparations often just one enzyme is used in the cells. If multistep reactions are to be carried out some different possibilities appear. One is to co-immobilize enzymes which carry out the different steps in the sequence. Immobilized multistep enzyme systems have been studied extensively by Mattiasson (1974). Another possibility is to use a sequence of enzymes in whole organelles or whole cells. In the literature there are many examples of these types of biocatalysts (Birnbaum et al. 1983, Mattiasson 1983a). Sometimes it is beneficial to co-immobilize an enzyme with whole cells or to co-immobilize different kinds of cells (Hahn-Hägerdal 1983).

The reason for using a co-immobilized preparation is that the product of one cell type is utilized by the other cell type. In paper III the oxygen produced by the algae was effectively used by the bacteria in the co-immobilized preparation. Since the distance between the algal and the bacterial cells in the gel was quite short, mass transfer of oxygen was rapid. Little oxygen escaped from the gel particles. One can imagine situations where both cell types obtain substrates from the other cell type. One example would be the co-immobilization of algae with organisms that produce carbon dioxide as well as consuming

oxygen. In this case a situation similar to that in natural symbiotic organisms appears. One example of natural symbionts of this type is the lichens which consist of algae and fungi.

One risk with using co-immobilized living cells is that one cell type may produce substances that are harmful to the other components of the preparation. It is therefore important to make the right choice of organisms for the co-immobilized preparation. Most reactions can be carried out by many different species of organisms so it ought to be possible to find acceptable combinations to carry out most multistep reactions. In paper III no negative effects on the cells were noted. However, it has been reported that Chlorella algae are able to produce an antibacterial substance called chlorellin (Robertson et al. 1944). The active substance has not been characterized but it is thought to be an oxidized lipid (Spoehr et al. 1949).

4.2.2. Decomposition of hydrogen peroxide

Hydrogen peroxide is decomposed to oxygen and water by catalase as well as by some inorganic catalysts. This reaction has been used to supply oxygen to oxygen-demanding enzymes or cells. These applications were recently reviewed by Holst (1984). Schlegel (1977) used hydrogen peroxide as the oxygen source for the growth of several microorganisms at moderate cell densities. Bovine liver catalase was added to the cell suspensions. Provided that the rate of H_2O_2 addition was equal to the oxygen consumption rate of the cells, the cells grew at a rate almost identical to that under air. However, when bacterial suspensions of high cell densities were used, "aeration" with H_2O_2 caused cell damage and retardation of growth (Ibrahim and Schlegel 1980).

Hydrogen peroxide has also been used to supply oxygen to immobilized cell preparations. Holst et al. (1982) used this approach in the conversion of glycerol to DHA by immobilized G. oxydans cells. Hydrogen peroxide caused very high initial reaction rates, but the activity of the cells gradually decreased.

One plausible way of decreasing the harmful effects of hydrogen peroxide is to control its concentration and keep it at a low level. For this purpose a flow injection analysis (FIA) system was developed where hydrogen peroxide was detected amperometrically (Lundbäck et al. 1983). This analysis system was used for bioconversions with G. oxydans cells (Holst et al. in press). The signal from the FIA system was used to control the additions of hydrogen peroxide. The concentration of hydrogen peroxide was kept at constant levels between 0.5 and 1.0 mM. Even at the lowest of the hydrogen peroxide concentrations a marked decrease in oxygen consumption and cell viability was noted after 20 h of operation. Different techniques of increasing the stability of the cells have been discussed by Holst et al. (in press).

When hydrogen peroxide is used as the oxygen source, the mass transfer conditions are quite different from those when air is used. Since no gas phase is involved there is no gas-liquid mass transfer resistance (steps 1 and 2 in Fig 4). Hydrogen peroxide is very soluble in water so high concentrations can be used in the bulk liquid phase which leads to decreased external and internal mass transfer resistance in the immobilized preparations (steps 4 and 5 in Fig 4). However, if low concentrations of hydrogen peroxide have to be used (to improve the stability of the cells) external and internal mass transfer will increase in importance. Mass transfer of oxygen from catalase to the oxygen consuming sites of the cells is rapid under most conditions.

It is important that oxygen production and consumption are balanced. If oxygen production is too slow the productivity will be low and if it is much faster than the oxygen consumption, gas bubbles form and these can disrupt the matrix. This balance can be controlled via the concentration of hydrogen peroxide and the catalase content if catalase is co-immobilized with the cells.

4.2.3. Electrolytic generation of oxygen

Electrolytic decomposition of water has been used in order to supply oxygen to fermentations of Pseudomonas fluorescens (Sadoff et al. 1956). Under some experimental conditions toxic quantities of hypochlorite were formed. In complex reactant solutions many side reactions can occur at the electrodes thus limiting the use of this method.

Electrolytic decomposition of water to increase oxygen supply to immobilized enzymes was used by Enfors (1981) in the construction of an enzyme electrode for glucose analysis. Glucose oxidase and catalase were immobilized on a Pt-gauze which was connected as an anode in an electrolysis circuit. The electrolysis current was controlled by the differential potential between the oxygen sensing part of the glucose electrode and a reference voltage. The electrolysis current gave a measure of the concentration of glucose surrounding the enzyme electrode. The glucose electrode has been used to measure the glucose concentration continuously in batch fermentations of Candida utilis (Enfors 1981) and in glucose-fed batch cultivations of E. coli (Cleland and Enfors 1983).

4.3. Oxygen carriers

One approach to increasing the oxygen supply to processes with immobilized biocatalysts is to modify the reactant solution so that it can bring more oxygen into the reactor. Different kinds of oxygen carrying substances can be added to the reactant solution. The most straightforward way is to replace water, which is normally used in bioconversions, by another solvent that dissolves more oxygen. In these cases oxygen is dissolved according to Henry's law (eq (1), Fig. 9a). Accordingly the oxygen concentration in the liquid can be greatly increased by increasing the partial pressure of oxygen in the gas phase by using, for example, pure oxygen instead of air to saturate the liquid.

Some substances are capable of specific oxygen binding. In nature oxygen binding proteins such as hemoglobin and myoglobin are found. Some synthetic metal complexes also bind oxygen reversibly. For this specific binding Henry's law does not apply. Only a certain number of oxygen binding sites are available. The saturation curve of an oxygen carrier of this kind is shown in figure 9b.

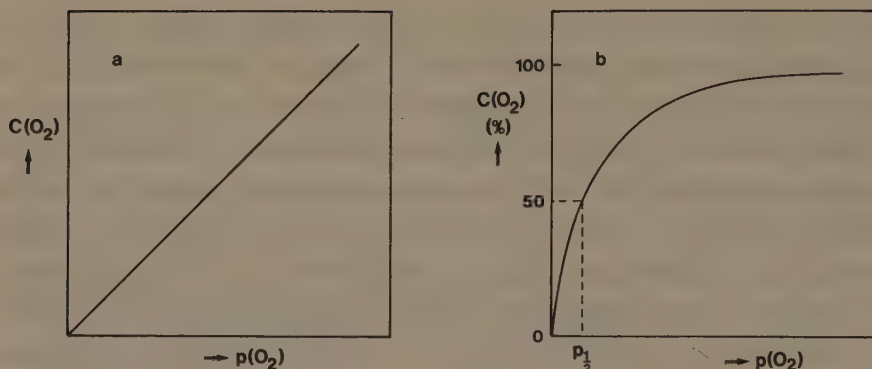


Fig 9. The oxygen concentration in the liquid phase at equilibrium as a function of the partial pressure of oxygen in the gas phase. Oxygen saturation according to Henry's law (a) and for a specific oxygen carrier such as myoglobin (b).

An oxygen carrier must fulfil certain requirements in order to be useful in biotechnical processes:

- 1) Reversible oxygenation
- 2) Suitable $p_{1/2}$ value (see below)
- 3) Stability under the conditions used for the process
- 4) Low toxicity
- 5) Low price

An important characteristic of a specific oxygen carrier is the $p_{1/2}$ value, which is the oxygen pressure at which the carrier is half saturated with oxygen. If the $p_{1/2}$ value is too high, it is difficult to saturate the carrier with oxygen and if it is too low it is difficult to remove oxygen from the carrier. Biocatalysts with high K values (low oxygen affinity) must have carriers with high $p_{1/2}$ values while biocatalysts with low K values (high oxygen affinity) can also use oxygen carriers with low $p_{1/2}$ values.

The carrier should of course be stable under the conditions during the process. However, the carrier is often slowly oxidized by oxygen. Oxygen carrying proteins are usually nontoxic. However, synthetic metal complexes might be toxic but little information about the toxicity of oxygen binding complexes is available. Finally, the cost of the oxygen carrier must not be too high.

When used to increase the oxygen supply to immobilized biocatalysts, oxygen carriers are often recirculated through a reactor containing the immobilized preparation. Between the passages through the reactor the oxygen carrier is oxygenated and in the reactor oxygen is unloaded to support the oxygen demanding process. Either the complete reaction medium or just the oxygen carrier can be recirculated. The use of a separate oxygenator is often advantageous because the immobilized preparation is often sensitive to vigorous stirring which is essential to achieve good gas-liquid oxygen transfer.

Compared to the recirculation of a water solution without an oxygen carrier through the reactor, the use of oxygen carriers make it possible to use lower flow rates and fewer passages through the reactor are needed to achieve a certain product concentration.

4.3.1. Use of organic solvents

Water is a poor solvent for oxygen. The solubility of oxygen in many other liquids is considerably higher (Table 2).

Solvent	oxygen solubility (mM)	T(°C)	
water	1.26	25	
water	2.00	4	1)
hexadecane	5.1	18	
ethanol	6.38	19.8	
benzene	7.3	19	
toluene	7.5	18	
chloroform	9.15	16.3	
carbon tetrachloride	10.3	17.8	
petroleum ether (60-80°C)	18.3	18.5	
ether	18.5	20.3	
FC-72	26.6	25	2)

Table 2. Oxygen solubility in various liquids. Mainly from Buckland et al. (1975). 1) Montgomery et al. (1964). 2) 3M (1979).

Buckland et al. (1975) used additions of organic solvents in the oxidation of cholesterol to cholestanon. Their intention was to increase both the solubility of the steroids and oxygen. The conversion degree was 3 % in water and 24 % in toluene.

Organic solvents are often harmful to biocatalysts. Therefore the use of organic solvents for bioconversions has been limited although some examples can be found in the literature (Lilly 1982). The choice of organic solvent is critical.

4.3.2. Perfluorochemicals and silicon compounds

Perfluorochemicals are organic compounds where all hydrogen atoms are exchanged by fluorine atoms (Fig. 10). These compounds are remarkably stable to heat and chemical reagents. They are nonpolar and their solubility in water is very low. However, the solubility of many gases, for example oxygen and carbon dioxide, is quite high in perfluorochemicals.

The high solubility of oxygen and carbon dioxide in perfluorochemicals along with their inertness make them attractive as blood substitutes. Emulsions of perfluorochemicals have been used as blood substitutes for animals (Riess and LeBlanc 1978) and humans (Lowe 1984, Tremper 1985). A commercial product, fluosol DA, is manufactured in Japan for use as a blood substitute.

Silicon compounds, such as polydimethylsiloxane, are also nonpolar and chemically inert. Like perfluorochemicals they dissolve high concentrations of gases such as oxygen and carbon dioxide.

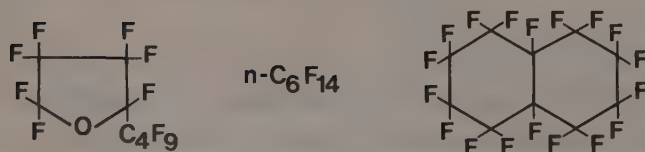


Fig 10. Some examples of perfluorochemicals.

4.3.2.1. Use of perfluorochemicals and silicon compounds as oxygen carriers in biotechnology

Chibata et al. (1974) in a patent described the addition of perfluorochemicals and silicon compounds to aerobic fermentations. The fermentations were carried out in shake flasks or gas sparged stirred tank fermentors. However, according to the calculations of Damiano and Wang (1985), the productivity improvements were small.

In paper IV the use of emulsions of the perfluorochemical FC-72 (produced by 3M) in the conversion of glycerol to DHA by immobilized G. oxydans cells is described. The addition of FC-72 had a marked positive effect on the productivity. As the dissolution of oxygen in perfluorochemicals obeys Henry's law, the use of pure oxygen instead of air for the saturation of the reactant solution increased the productivity by a factor of 4-5. The cells retained the same level of viability when stored in the perfluorochemical emulsion as in the buffer. Moreover, the operational stability in the emulsion was good.

Another way of using perfluorochemicals for oxygenation was recently presented by Damiano and Wang (1985). Pure perfluorochemical (perfluoromethyldecalin) was saturated with air in a separate aerator. It was then dispersed into small droplets by a nozzle in the top of the fermentor. The droplets slowly fell through the aqueous phase to the bottom of the fermentor where they coalesced. The perfluorochemical was taken back to the aerator via a phase separator. Little energy was needed to form the droplets and the oxygen transfer from the droplets to the aqueous phase was rapid. This new aeration system was successfully used in cultivations of E. coli.

Leonhardt et al. (1985) used emulsions of perfluorodecalin or silicon compounds as oxygen carriers for Providencia cells immobilized in calcium alginate. In contrast to Damiano and Wang (1985) they did not find perfluorochemicals suitable for supplying oxygen to growing cells. Better cell growth was obtained with polydimethylsiloxane emulgated with a copolymer of polydimethylsiloxane and polyethylene oxide. Furthermore when the production of α -ketoacid from L-aminoacid (L-methionine) by Providencia cells was measured, silicon emulsions were more efficient than emulsions of perfluorochemicals.

4.3.3. Oxygen carrying proteins

In nature some different oxygen carrying proteins are found. The most well known are hemoglobin and myoglobin. In both these proteins oxygen is reversibly coordinated to the iron atoms of the heme groups. Myoglobin binds oxygen strongly. The saturation curve is hyperbolic and the $p_{1/2}$ value is about 1 torr (Stryer 1975). Hemoglobin, which binds oxygen less strongly, has a $p_{1/2}$ value of about 26 torr. Its oxygen saturation curve is sigmoidal due to the positive cooperativity between the subunits. The oxygen affinity is regulated by 2,3-diphosphoglycerate which lowers the oxygen affinity when it binds with the hemoglobin molecule.

Hemoerythrin is a non-heme iron protein which is an oxygen carrier in some animals. In this case no prosthetic group seems to be present but the iron atom is bound directly to the protein. Some animals use hemo-cyanins as their oxygen carrying protein. In these proteins copper is responsible for the oxygen binding capacity. Moreover vanadin-containing oxygen carrying proteins have also been found (Bayer and Schretzmann 1967).

4.3.3.1. Use of oxygen carrying proteins

Several blood substitutes based on hemoglobin have been developed. If solutions of hemoglobin are given as blood substitutes to humans, hemoglobin is rapidly excreted via the kidneys. Tam (1979) used soluble dextran-hemoglobin complexes and Ajisaka and Iwashita (1980) used polyethylene glycol modified hemoglobin as blood substitutes. By these different modifications hemoglobin was kept in the circulation system for a long time. Djordjevic (1979) encapsulated hemoglobin in liposomes. These liposomes resembled erythrocytes and were successfully used in blood transfusions to animals.

Mattiasson and Borrebaeck (1978) used erythrocytes to increase the oxygen supply to immobilized glucose oxidase and thereby improved the performance of their glucose analysis system.

Different forms of immobilized hemoglobin have been used to bind oxygen reversibly. Bonaventura et al. (1984) attempted to extract oxygen from seawater and use the oxygen to support underwater life, for example in submarines. Hemoglobins from different sources were tried but the immobilized forms had an oxygen affinity that was too high. By replacing the iron atoms in hemoglobin by cobalt atoms, coboglobin with lower oxygen affinity was obtained. Coboglobin immobilized on silica had a p_{50} value of about 40 torr which was considered suitable. The system showed good reversibility.

In paper IV solutions of hemoglobin were used to supply oxygen to immobilized G. oxydans cells in a packed bed reactor. The cells had sufficiently high affinity for oxygen to extract it from hemoglobin. The production of DHA was greatly increased by the addition of hemoglobin. When the hemoglobin solutions were recirculated through the reactor for long periods of time their oxygen transporting capacity decreased due to the oxidation of hemoglobin to methemoglobin. Methemoglobin, which contains Fe(III) instead of Fe(II), is not able to bind oxygen. At pH 5 the oxidation of hemoglobin was rapid so that the production of DHA had practically ceased after a few hours. It is known that hemoglobin is more stable towards oxidation at higher pH values (Mansouri and Winterhalter 1973). In the present example pH 6 was optimal. At still higher pH values the bacteria were not so active although hemoglobin was stable. The oxidation of hemoglobin can be reversed. Different reducing reagents such as ascorbic acid (Tomoda et al. 1976) and iron(II)EDTA-complex (Mauk and Gray 1979) have been used. In vivo, methemoglobin is reduced enzymatically. A purified NADPH-dehydrogenase from human erythrocytes has been used in vitro to reduce methemoglobin in the presence of a flavin (Yubisui et al. 1977).

Another potential problem with the use of hemoglobin in supplying oxygen to immobilized cells is that the protein part of hemoglobin might be attacked by proteases from the cells. In paper IV no such effects were observed.

In conclusion hemoglobin can be a good oxygen carrier for immobilized biocatalysts provided the reaction takes place at neutral or alkaline pH.

Oxygen carrying proteins can also be used to remove oxygen from oxygen inhibited processes. In the root nodules of legumes, leghemoglobin is present. This hemoglobin binds oxygen and thereby probably protects the Rhizobia in the root nodules. The nitrogen fixing enzyme system of the Rhizobia is inhibited by oxygen. The $p_{1/2}$ value of leghemoglobin from soybean is as low as 0.04 torr (Appleby 1962).

Rosenkrans and co-workers used hemoglobin and myoglobin to remove oxygen and thereby increase the hydrogen photoproduction of Scenedesmus algae (Rosenkrans et al. 1983, Rosenkrans and Krasna 1984). Hemoglobin and myoglobin served as biocompatible oxygen carriers. The hydrogen production was further increased when other strong oxygen binding reagents were present in a separate chamber in the test apparatus. These reagents, such as the synthetic cobalt chelates in DMSO solution, were often not biocompatible.

4.3.4. Metal complexes

Among oxygen carriers synthetic metal complexes have been studied extensively. Although such complexes have not to any great extent been used as oxygen carriers in biotechnology they are of considerable potential interest and some of the research in this field will therefore be briefly reviewed.

A good general review about reversible oxygenation of metal complexes was written by Bayer and Schretzmann (1967). Additional information can be found in the review of Erskine and Field (1976). Most of the oxygen carrying complexes studied have contained Fe(II) (Herron et al. 1983) or Co(II) (Nakon and Martell 1972, McLendon et al. 1975) but other metal ions have also been used in such complexes. Metal complexes with a wide range of oxygen affinities have been prepared.

The main problem with the use of synthetic metal complexes as oxygen carriers is that the metals of the complexes are often oxidized by oxygen. The resulting oxidized complexes cannot transport oxygen. As pointed out earlier the same problem occurs with oxygen carrying proteins. In the solid state some metal complexes can be oxygenated and deoxygenated several thousand times (Bayer and Schretzmann 1967).

Many of the metal complexes are also stable to oxidation in anhydrous solvents especially at low temperatures. In aqueous solutions oxidation of the metal occurs. This oxidation is catalyzed by hydrogen atoms. If the complexes are to be used in an aqueous environment it is sometimes possible to minimize the oxidation of the metal by creating a hydrophobic microenvironment around the metal complex. This approach was used when derivatized heme groups were embedded in polystyrene (Wang 1958). This preparation also showed reversible oxygenation in aqueous media since water was excluded from the vicinity of the heme groups by polystyrene.

4.4. Use of artificial electron acceptors

For the oxidases oxygen is a good electron acceptor. However, it is known that most oxidases can also use other electron acceptors such as 2,6-dichlorophenolindophenol or ferricyanide (Barman 1969). In the same way many dehydrogenases can use these artificial electron acceptors.

Dixon (1971) reviewed data on the acceptor specificity of flavo-enzymes. He did not find any acceptors that worked with all the enzymes. Furthermore he found it impossible to make any good correlations between the acceptor specificity and other properties of the flavo-enzymes. The possible correlations investigated included redox potential, reaction mechanism, nature and number of flavin groups and occurrence of metal atoms in the enzyme.

Although artificial electron acceptors are well known in enzymology they have been used only to a minor extent for preparative purposes. Some artificial electron acceptors have been used to increase the rate of steroid dehydrogenation reactions. Stefanovic et al. (1963) tested several electron acceptors for the Δ^1 -dehydrogenation of 4-androstenedione by cell free preparations of Bacillus sphaericus. Phenazine methosulfate and menadione were the most efficient acceptors. Ohlson et al. (1978) studied the Δ^1 -dehydrogenation of hydrocortisone by Arthrobacter simplex cells. They found that the productivity was markedly enhanced when menadione was added to the reactant solution. However, menadione caused a significant drop in operational stability. Similar observations were made by Manecke and Beier (1983).

Alberti and Klivanov (1982) showed that p-benzoquinone can replace oxygen as the electron acceptor for glucose oxidase. In the reaction p-benzoquinone was reduced to hydroquinone. A column with immobilized glucose oxidase was used continuously for two weeks for the production of hydroquinone by this reaction.

In paper V p-benzoquinone was used as an artificial electron acceptor in the oxidation of glycerol to DHA by G. oxydans cells (Fig. 11). The concentration of p-benzoquinone that gave half of the maximal reaction rate was 3-4 mM. The maximal reaction rate obtainable with p-benzoquinone as the electron acceptor was higher than the rate with oxygen. In an experiment with free cells p-benzoquinone gave a rate more than four times that of oxygen and with immobilized cells the difference was even greater. In addition to the fact that p-benzoquinone gives a higher maximal reaction rate, it is advantageous that high concentrations of p-benzoquinone can be used since this facilitates the mass transfer of the electron acceptor. This is a major advantage when immobilized cells are used. There are also factors that favour the use of oxygen. The cells have a higher affinity for oxygen (lower K value) than for p-benzoquinone. Oxygen is also a smaller molecule that diffuses more rapidly through the matrix than p-benzoquinone. However, these factors are apparently less important than the factors favouring the use of p-benzoquinone.

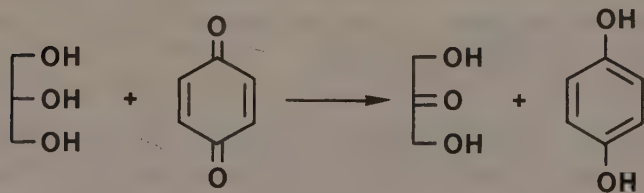


Fig 11. Oxidation of glycerol to DHA with p-benzoquinone as the electron acceptor.

The operational stability was comparatively good. In one experiment the productivity decreased from 60 to 10 mmol/h·g over an 8-day period when p-benzoquinone was used. When oxygen was used in a similar experiment the productivity decreased from 14 to 6 mmol/h·g. The by-product formed from p-benzoquinone, hydroquinone, can be oxidized

to p-benzoquinone which can be reused. In paper V hydroquinone was oxidized with hydrogen peroxide and immobilized peroxidase (Fig. 12). Seven successive regenerations of p-benzoquinone were performed without any loss of efficiency.

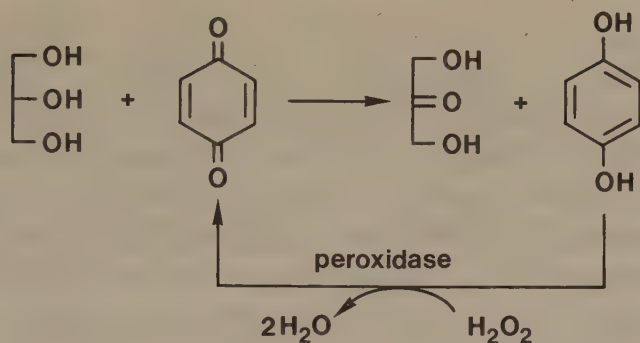


Fig 12. Oxidation of glycerol to DHA with regeneration of the electron acceptor p-benzoquinone.

It is interesting that a higher reaction rate can be obtained with p-benzoquinone than with the natural electron acceptor, oxygen. The rate limiting step in the normal catalytic reaction seems to be the reoxidation of the glycerol dehydrogenase by the electron transport chain. Probably p-benzoquinone can reoxidize the enzyme itself or some component of the electron transport chain so that the rate limiting step is avoided and a higher overall rate is achieved.

Furthermore in other oxidation reactions, p-benzoquinone has been used as the electron acceptor. In both the oxidation of sorbitol to sorbose and of glucose to gluconic acid by G. oxydans a higher reaction rate was achieved with p-benzoquinone than with oxygen (Adlercreutz unpublished). Moreover, in the oxidation of glycerol to DHA by Acetobacter aceti (ATCC 23769) p-benzoquinone gave a higher reaction rate than oxygen. However, p-benzoquinone was not effective as an electron acceptor in the oxidation of methanol by Hansenula yeast. In the dehydrogenation of hydrocortisone to prednisolone by Arthrobacter simplex p-benzoquinone was a good electron acceptor, although it was not as efficient as phenazine methosulfate at low acceptor concentrations (Adlercreutz and Mattiasson 1985).

5. CONCLUDING REMARKS

Several methods for improving the oxygen supply to immobilized biocatalysts have been presented. They all have their special characteristics which make them suitable for different applications.

The methods are useful in different pH intervals. The oxygen production by algae (*C. pyrenoidosa*) had a pH optimum of 7. Other algal species might have different pH optima. Among the oxygen carriers, hemoglobin is not suitable at low pH values because it is oxidized to methemoglobin. Synthetic metal complexes are also less stable at low pH values. However, perfluorochemical emulsions can be used in a wide pH range. Decomposition of hydrogen peroxide and the use of artificial electron acceptors are also rather insensitive to variations in pH.

Most bioconversions are performed at moderate temperatures. However, when thermostable enzymes are to be used it will probably be advantageous to carry out the bioconversions at high temperatures. Most algae do not work well at high temperatures and oxygen carrying proteins might denature while the other methods are generally less sensitive to elevated temperatures.

One important point worth considering when increasing the oxygen supply to immobilized biocatalysts is the operational stability. There are in principal two reasons why productivity can decrease during the operation of the process. One is that the biocatalyst loses its activity by itself or through the oxygen supply system. The other reason is that the efficiency of the oxygen supply system might decrease during operation.

All the methods of increasing the oxygen supply may potentially cause a decrease in the activity of the biocatalyst. Hydrogen peroxide and its reaction products are harmful to many biocatalysts. Artificial electron acceptors are often reactive substances that one has reason to believe are dangerous to living cells. Some algae produce substances that inhibit other organisms. Perfluorochemicals are often quite biocompatible but if they are used as emulsions the emulsifier might harm the biocatalyst. Oxygen carrying proteins are probably harmless to most biocatalysts while synthetic metal complexes might be toxic.

The stability of the oxygen supply system is of great importance. Algae seem to stand co-immobilization with bacteria quite well. In the co-immobilized preparation the algae multiplied and no decrease in productivity was observed (paper III). When decomposition of hydrogen peroxide is used to generate oxygen problems might arise because of a decreased catalase activity. Oxygen carrying proteins are not perfectly stable. The metal ion is often oxidized by oxygen which also occurs in synthetic metal complexes. In oxygen carrying proteins the protein part of the molecule can be attacked by proteases. Artificial electron acceptors are normally capable of reacting with many cell components and they can therefore be withdrawn from their function as electron acceptors.

The mass transfer resistance for oxygen is decreased to a variable extent by the different methods. In a co-immobilized algae-bacteria preparation mass transfer of oxygen is rapid because of the short distance between the cells. Mass transfer of carbonate, the substrate of the algae, did not limit the reaction rate (paper III). When oxygen is generated in situ from hydrogen peroxide, oxygen transfer is also rapid. Depending on the concentration of hydrogen peroxide the diffusion of this substance can influence the reaction rate.

Large oxygen carriers, such as droplets of organic solvents, cannot enter the biocatalyst. They unload oxygen outside the catalyst particles and oxygen is transported by external and internal diffusion to the oxygen demanding entities in the catalyst. Smaller oxygen carriers can enter the biocatalyst and unload oxygen inside the matrix near the enzymes or cells. In this way both external and internal mass transfer is facilitated.

By using high concentrations of artificial electron acceptors the mass transfer resistance can be greatly reduced. However, it is not always possible to use these high concentrations of electron acceptors.

It is obviously not possible to say which of the methods of improving oxygen supply is the best one. For each application many factors must be considered before a proper choice can be made. Sometimes it might be advantageous to use a combination of different methods.

A model system consisting of G. oxydans cells immobilized in calcium alginate was used in papers I and III-VI and also in other studies (Holst et al. 1982, Holst et al. in press) to evaluate methods of increasing the oxygen supply to immobilized biocatalysts. Although it was not the aim of this work to develop a process for the production of DHA with G. oxydans cells, it is interesting to compare the results achieved with the different methods. The two most important criteria are the productivity and the operational stability. Decomposition of hydrogen peroxide gave a high initial reaction rate but the operational stability was poor (Holst et al. 1982). Good operational stability was obtained with the co-immobilized algae-bacteria preparation (paper III) and with perfluorochemical emulsions as oxygen carriers (paper IV). However, these methods did not give such a high specific productivity. As a goal for the specific productivity of immobilized cells one can use the specific productivity of free cells. At 30°C this productivity was 75 mmol/g·h (Table 3). In normal immobilized preparations considerably lower productivities were obtained. However with p-benzoquinone (40 mM) as the electron acceptor a productivity of 109 mmol/g·h was achieved and the operational stability was comparable to that obtained with oxygen. A comparison with the rate obtained with free cells and p-benzoquinone as the electron acceptor, 350 mmol/g·h, shows that mass transfer still limits the reaction rate. Maybe it is possible to find an electron acceptor with properties even more suitable than those of p-benzoquinone.

Productivity (mmol DHA/g·h)			
p-benzoquinone (40 mM)		oxygen	
Free cells	350	air saturated	75
		oxygen saturated	75
Immobilized cells	109	air saturated	6.4
($r_0 = 1.1 \text{ mm}$, $\rho = 28 \text{ kg/m}^3$)		oxygen saturated	18

Table 3. Productivity of G. oxydans cells converting glycerol to DHA with oxygen or p-benzoquinone as the electron acceptor.

One possibility of overcoming the mass transfer resistance is to use another immobilization technique. Immobilization on the surface of particles would eliminate much of the mass transfer resistance but such immobilization is often more harmful to the cells than entrapment in calcium alginate and with surface immobilization it is more difficult to obtain high cell densities.

So far, few full-scale processes involving immobilized cells with a high oxygen demand have been developed. This thesis has pointed out some of the ways of treating the problem of oxygen supply to such processes.

6. LIST OF SYMBOLS

c_{bs}	bulk substrate concentration, kmol/m^3
c_{is}	internal substrate concentration, kmol/m^3
c_{os}	substrate concentration at particle surface, kmol/m^3
D	external substrate diffusivity, m^2/s
D_{is}	internal substrate diffusivity, m^2/s
DHA	dihydroxyacetone
k_2	kinetic constant, $\text{kmol/s}\cdot\text{kg}$
K	Michaelis-Menten constant, kmol/m^3
k_C	mass transfer coefficient for external mass transfer, m/s
$p_{1/2}$	oxygen pressure at which the oxygen carrier is half saturated, torr
r	distance coordinate, m
r_0	particle radius, m
v_{free}	reaction rate of free cells, $\text{kmol/m}^3\cdot\text{s}$
v_{loc}	local reaction rate, $\text{kmol/m}^3\cdot\text{s}$
v_{max}	maximal reaction rate, $\text{kmol/m}^3\cdot\text{s}$
v_{obs}	observed reaction rate, $\text{kmol/m}^3\cdot\text{s}$
θ	porosity
η_0	overall effectiveness factor for catalyst particle
ρ	cell density, kg/m^3
Φ	Thiele modulus

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8. REFERENCES

Adachi O, Tayama K, Shinagawa E, Matsushita K and Ameyama M (1978) *Agric Biol Chem* 42, 2045.

Adachi O, Tayama K, Shinagawa E, Matsushita K and Ameyama M (1980) *Agric Biol Chem* 44, 503.

Adlercreutz P and Mattiasson B (1982) *Acta Chem Scand B* 36, 651.

Adlercreutz P and Mattiasson B (1985) In: *Biocatalysts in organic synthesis, Symposium of the Working Party on Immobilized Biocatalysts of the European Federation of Biotechnology*, abstracts, p 18.

Ajisaka K and Iwashita Y (1980) *Biochem Biophys Res Commun* 97, 1076.

Alberti B N and Klivanov A M (1982) *Enzyme Microb Technol* 4, 47.

American Society for Testing and Materials (1980) *Annual book of ASTM standards*, part 31, p 538.

Ameyama M, Matsushita K, Ohno Y, Shinagawa E and Adachi O (1981a) *FEBS Lett* 130, 179.

Ameyama M, Shinagawa E, Matsushita K and Adachi O (1981b) *Agric Biol Chem* 45, 851.

Ameyama M, Shinagawa E, Matsushita K and Adachi O (1981c) *J Bacteriol* 145, 814.

Appleby C A (1962) *Biochim Biophys Acta* 60, 226.

Asai T (1968) *Acetic acid bacteria; Classification and biochemical activities*, Univ of Tokyo Press.

Atkinson B and Mavituna F (1983) *Biochemical Engineering and Biotechnology Handbook*, The Nature Press, New York.

Baker C A and Claus G W (1983) *FEMS Microbiology Lett* 18, 123.

Barman T E (1969) *Enzyme Handbook*, Vol 1, Springer-Verlag, Berlin and Heidelberg.

Bayer E and Schretzmann P (1967) *Structure and Bonding* 2, 181.

Bello R A, Robinson C W and Moo-Young M (1985) *Biotechnol Bioeng* 27, 369.

Birnbaum S, Larsson P-O and Mosbach K (1983) In: *Solid phase biochemistry* (Scouten H ed) p 679, J Wiley & Sons, New York.

Birnbaum S, Pendleton R, Larsson P-O and Mosbach K (1981) *Biotechnol Lett* 3, 393.

Boersma J G, Vellenga K, de Wilt H G J and Joosten G (1979) *Biotechnol Bioeng* 21, 1711.

Bonaventura C, Bonaventura J, Hooper I R and Marshall T (1984) *Appl Biochem Biotechnol* 9, 65.

- Buckland B C, Dunnill P and Lilly M D (1975) *Biotechnol Bioeng* 17, 815.
- Bungay H R, Whalen W J and Sanders W M (1969) *Biotechnol Bioeng* 11, 765.
- Calderbank P H and Moo-Young M B (1961) *Chem Eng Science* 16, 39.
- Cheetham P S J (1980) In: *Topics in Enzyme and Fermentation Biotechnology* (Wiseman A ed) Vol 4, p 189, Ellis Horwood Ltd, Chichester, England.
- Cheetham P S J (1983) In: *Principles of Biotechnology* (Wiseman A ed) p 172, Surrey Univ Press, London.
- Chibata I, Yamada S, Wada M, Izuo N and Yamaguchi T (1974) U S Patent 3,850,753.
- Cleland N and Enfors S-O (1983) *Eur J Appl Microbiol Biotechnol* 18, 141.
- Damiano D and Wang S S (1985) *Biotechnol Lett* 7, 81.
- De Ley J, Gillis M and Swings J (1984) *Acetobacteraceae*. In: *Bergey's Manual of Systematic Bacteriology* (Krieg N R and Holt J G eds) Vol 1, p 267, Williams and Wilkins, Baltimore and London.
- Dixon M (1971) *Biochim Biophys Acta* 226, 269.
- Djordjeovich L (1979) *Diss Abstr Int B* 40, 2281.
- Duine J A, Frank J and van Zeeland J K (1979) *FEBS Lett* 108, 443.
- Enfors S-O (1981) *Enzyme Microb Technol* 3, 29.
- Enfors S-O and Mattiasson B (1983) In: *Immobilized cells and organelles* (Mattiasson B ed) Vol II, p 41, CRC Press, Boca Raton, FL.
- Engasser J-M and Horvath C (1973) *J Theor Biol* 42, 137.
- Erskine R W and Field B O (1976) *Structure and Bonding* 28, 1.
- European Federation of Biotechnology (1983) *Enzyme Microb Technol* 5, 304.
- Fink D J, Na T-Y and Schultz J S (1973) *Biotechnol Bioeng* 15, 879.
- Ghommidh C, Navarro J M and Durand G (1982) *Biotechnol Bioeng* 24, 605.
- Goldstein L (1976) *Methods Enzymol* 44, 397.
- Hackel U, Klein J, Megnet R and Wagner F (1975) *Eur J Appl Microbiol* 1, 291.
- Hahn-Hägerdal B (1983) In: *Immobilized cells and organelles* (Mattiasson B ed) Vol II, p 79, CRC Press, Boca Raton, FL.

- Hamilton B K, Gardner C R and Colton C K (1974) A I Ch E Journal 20, 503.
- Herron N, Zimmer L L, Grzybowski J J, Olszanski D J, Jackels S C, Callahan R W, Cameron J H, Cristoph G G and Bush D H (1983) J Am Chem Soc 105, 6585.
- Hiemstra H, Dijkhuizen L and Harder W (1983) Eur J Appl Microbiol Biotechnol 18, 189.
- Holst O (1984) Improved oxygen supply to immobilized cells by in situ oxygen generation, Thesis, Univ of Lund.
- Holst O, Enfors S-O and Mattiasson B (1982) Eur J Appl Microbiol Biotechnol 14, 64.
- Holst O, Lundbäck H and Mattiasson B (in press) Appl Microbiol Biotechnol.
- Ibrahim M and Schlegel H G (1980) Biotechnol Bioeng 22, 1877.
- Kasche V (1983) Enzyme Microb Technol 5, 2.
- Kasche V and Kuhlmann G (1980) Enzyme Microb Technol 2, 309.
- Kennedy J F and Cabral J M S (1983) In: Solid phase biochemistry (Scouten W H ed) p 253, J Wiley & Sons, New York.
- Kierstan M and Bucke C (1977) Biotechnol Bioeng 19, 387.
- Klein J, Stock J and Vorlop K-D (1983) Eur J Appl Microbiol Biotechnol 18, 86.
- Klein J and Washausen P (1979) In: Characterization of Immobilized Biocatalysts (Buchholz K ed) p 300, Verlag Chemie, Weinheim and New York.
- Kobayashi T and Laidler K J (1973) Biochim Biophys Acta 302, 1.
- Laws E A, Terry K L, Wickman J and Chalup M S (1983) Biotechnol Bioeng 25, 2319.
- Lee Y-K and Pirt S J (1981) J Gen Microbiol 124, 43.
- Leonhardt A, Szwajcer E and Mosbach K (1985) Appl Microbiol Biotechnol 21, 162.
- Lilly M D (1982) J Chem Tech Biotechnol 32, 162.
- Lowe K C (1984) Pharm J 232, 73.
- Lundbäck H, Johansson G and Holst O (1983) Anal Chim Acta 155, 47.
- Makhotkina T A, Pomortseva N V, Lomova I E and Nikolaev P I (1981) Prikl Biokhim Mikrobiol 17, 102.
- Manecke G and Beier W (1983) Angew Macromol Chem 113, 179.
- Mansouri A and Winterhalter K H (1973) Biochemistry 12, 4946.

- Mattiasson B (1974) Studies on immobilized multi-step-enzyme systems, Thesis, Univ of Lund.
- Mattiasson B (ed) (1983a) Immobilized cells and organelles, CRC Press, Boca Raton, FL.
- Mattiasson B (1983b) In: Immobilized cells and organelles (Mattiasson B ed) Vol I, p 3, CRC Press, Boca Raton, FL.
- Mattiasson B and Borrebaeck C (1978) FEBS Lett 85, 119.
- Mauk A G and Gray H B (1979) Biochem Biophys Res Commun 86, 206.
- McLendon G, MacMillan D T, Hariharan M and Martell A E (1975) Inorg Chem 14, 2322.
- Montgomery H A C, Thom N S and Cockburn A (1964) J Appl Chem 14, 280.
- Nakon R and Martell A E (1972) Inorg Chem 11, 1002.
- Nilsson K (1984) A new immobilization method based on organic sulfonyl halides, Thesis, Univ of Lund.
- Ohlson S, Larsson P-O and Mosbach K (1978) Biotechnol Bioeng 20, 1267.
- Paul F and Vignais P M (1980) Enzyme Microb Technol 2, 281.
- Radovich J M (1985) Enzyme Microb Technol 7, 2.
- Regan D L, Lilly M D and Dunnill P (1974) Biotechnol Bioeng 16, 1081.
- Riess J G and Le Blanc M (1978) Angew Chem 90, 654.
- Robertson P, Daniels T C, Eiler J J, Gunnison J B, Kumler W D, Oneto J F, Strait L A, Spoehr H A, Hardin G J, Milner H W, Smith J H C and Strain H H (1944) Science 99, 351.
- Rosenkrans A M and Krasna A I (1984) Biotechnol Bioeng 26, 1334.
- Rosenkrans A M, Rosen M M and Krasna A I (1983) Biotechnol Bioeng 25, 1897.
- Sadoff H L, Halvorson H O and Finn R K (1956) Appl Microbiol 4, 164.
- Salisbury S A, Forrest H S, Cruse W B T and Kennard O (1979) Nature 280, 843.
- Sato K and Toda K (1983) J Ferment Technol 61, 239.
- Sato S, Mukataka S, Kataoka H and Takahashi J (1981) J Ferment Technol 59, 221.
- Schlegel H G (1977) Biotechnol Bioeng 19, 413.
- Schumpe A, Quicker G and Deckwer W-D (1982) Adv Biocem Eng 24, 1.
- Seiskari P, Linko Y-Y and Linko P (1985) Appl Microbiol Biotechnol 21, 356.

Shinagawa E, Matsushita K, Adachi O and Ameyama M (1981) Agric Biol Chem 45, 1079.

Shinagawa E, Matsushita K, Adachi O and Ameyama M (1982) Agric Biol Chem 46, 135.

Shinagawa E, Matsushita K, Adachi O and Ameyama M (1984) Agric Biol Chem 48, 1517.

Smidsröd O, Haug A and Whittington S G (1972) Acta Chem Scand 26, 2563.

Spoehr H A, Smith J H C, Strain H H, Milner H W and Hardin G J (1949) Carnegie Inst Wash Pub No 586, Chemical abstracts 44, 695.

Stefanovic V, Hayano M and Dorfman R I (1963) Biochim Biophys Acta 71, 429.

Stryer L (1975) Biochemistry, W H Freeman, San Francisco.

Takata I, Tosa T and Chibata I (1977) J Solid-Phase Biochem 2, 225.

Tam M S C (1979) Diss Abstr Int B 40, 735.

Tham M K, Walker R D and Gubbins K E (1970) J Phys Chem 74, 1747.

Tomoda A, Matsukawa S, Takeshita M and Yoneyama Y (1976) J Biol Chem 251, 7494.

Tramper J, Luyben K Ch A M and van den Tweel W J J (1983) Eur J Appl Microbiol Biotechnol 17, 13.

Tramper J, Suwinska-Borowiec G and Klapwijk A (1985) Enzyme Microb Technol 7, 155.

Tremper K (ed) (1985) Perfluorochemical oxygen transport. International Anesthesiology Clinics. Vol 23, no 1.

Tsukamoto T, Morita S and Okada J (1982) Chem Pharm Bull 30, 782.

Van Ginkel C G, Tramper J, Luyben K Ch A M and Klapwijk A (1983) Enzyme Microb Technol 5, 297.

Veliky I A and Williams R E (1981) Biotechnol Lett 3, 275.

Wang J H (1958) J Am Chem Soc 80, 3168.

Wikström P, Szwajcer E, Brodelius P, Nilsson K and Mosbach K (1982) Biotechnol Lett 4, 153.

Wittler R, Baumgärtl H, Schügerl K and Lübbers D W (1984) Third Eur Congr Biotechnol, abstracts, Vol I, p 513.

Yamané T, Araki S and Sada E (1981) J Ferment Technol 59, 367.

Yubisui T, Matsuki T, Tanishima K, Takeshita M and Yoneyama Y (1977) Biochem Biophys Res Commun 76, 174.

3M (1979) Fluorinert electronic liquids.

Characterization of *Gluconobacter oxydans* immobilized in calcium alginate

Patrick Adlercreutz¹, Olle Holst², and Bo Mattiasson¹

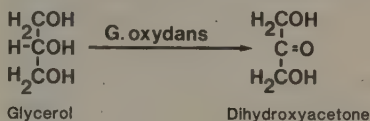
¹ Department of Biotechnology and ² Department of Applied Microbiology; Chemical Center, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden

Summary. *Gluconobacter oxydans* cells were immobilized in calcium alginate and the preparation was used for the oxidation of glycerol to dihydroxyacetone. The characterization was done according to the guidelines given by the Working Party on Immobilized Biocatalysts of the European Federation of Biotechnology. The pH optimum of the preparation was found to be 5.0 and the temperature optimum was 40°C. However, the operational stability was better at 30°C. The glycerol concentration required to obtain half the maximal reaction rate was about 5 mM for both immobilized and free cells. At low concentrations of glycerol and high concentrations of dihydroxyacetone a slight inhibition was noted. No loss of activity of the immobilized preparation was observed after storage for 68 days at +4°C. Investigation of the operational stability revealed a half-life of 5 days. Studies of the influence of particle size and cell densities as well as that of oxygen concentration revealed that the oxygen supply was the rate limiting step.

Introduction

The acetic acid bacterium *Gluconobacter oxydans* (ATCC 621) is able to perform incomplete oxidations of many different substrates. Several of the products formed are of commercial interest. The bacteria can, for example, oxidize glycerol to dihydroxyacetone (DHA) (Scheme 1). This reaction is done industrially with conventional fermentation technology (Green 1967). The use of immobilized cells would confer several advantages. However, when immobilized cells are used, ox-

ygen transfer from the bulk phase to the cells in the matrix limits the reaction rate (e.g. Tramper et al. 1983). Immobilized *G. oxydans* cells have been previously used as a model system to evaluate different methods of increasing the oxygen supply to immobilized cells (Holst et al. 1982, Adlercreutz et al. 1982, Adlercreutz and Mattiasson 1982, Adlercreutz and Mattiasson, 1984).



Some papers concerning the production of DHA with immobilized cells have already been published. Chibata and coworkers investigated the conversion of glycerol to DHA by *Acetobacter xylinum* immobilized in polyacrylamide gel (Nabe et al. 1979). The same conversion was studied by Makhotkina et al. (1981) with *G. oxydans* immobilized in polyacrylamide gel. Data on the use of this reaction as a model system when evaluating the use of hydrogen peroxide as an oxygen source for immobilized cells can be found in Holst et al. (1982).

The properties of *G. oxydans* immobilized in calcium alginate gel are presented here as determined according to the guidelines of the Working Party on Immobilized Biocatalysts of the European Federation of Biotechnology (European Federation of Biotechnology 1983).

Materials and Methods

Chemicals

Sodium alginate was purchased from Sigma (Type IV Lot No. 111F-0049) Dihydroxyacetone (98%) was supplied by Merck,

Darmstadt, FRG. All other chemicals were of analytical grade and obtained from commercial sources.

Organism

The bacteria *Gluconobacter oxydans* (ATCC 621) were cultivated as previously described (Holst et al. 1982). The amount of cells is always given as dry weight (Holst et al. 1982).

Immobilization

The cells were immobilized in calcium alginate gel as described elsewhere (Adlercreutz and Mattiasson, 1984). Preparations with varying particle sizes were obtained by extruding the cell-alginate slurry through hypodermic needles with varying diameters during immobilization. The diameters of the gel beads were measured with a microscope after storage overnight in a 0.1 M succinate buffer (pH 5.0) containing 10 mM CaCl_2 . The cell density of a certain preparation was calculated as the amount of cells immobilized (dry weight) divided by the volume of the beads. Immobilization of cells for determination of operational stability was carried out under sterile conditions.

Experimental set up for kinetic measurements

All the short-term experiments were carried out in a 115 ml reactor containing a polarographic oxygen electrode (Radiometer). The reactor was placed in a thermostated water bath and the solution was stirred magnetically. The magnet was fixed on an axis of stainless steel which passed through the top of the reactor through a hypodermic needle. In this way the solution in the reactor could be stirred without attrition of the alginate beads which takes place if ordinary magnetic stirring is used.

The buffer used in all experiments (except when pH was varied) was a 0.1 M succinate buffer (pH 5.0) containing 10 mM CaCl_2 . The buffer was poured into the reactor where it was thermostated and saturated with air or pure oxygen. The cells were then added as either free cells suspended in 0.9% NaCl-solution or immobilized cells and the lid, a rubber stopper, was put on.

In the experiments, 10 mg of free cells or 25 mg of immobilized cells (cell density: 29 g/l, particle diameter: 2.3 mm) were used and the temperature was 30°C if not otherwise stated.

The signal from the oxygen electrode was monitored and recorded. When the signal had stabilized, the substrate (glycerol) was added through a hypodermic needle. Usually glycerol was added to a final concentration of 0.25 M. The signal from the oxygen electrode was continuously recorded and the reaction rate could be calculated from the slope of the curve.

In most experiments, just the initial reaction rate was determined. However, as the oxygen concentration in the reactor decreased continuously, the reaction rate at different oxygen concentrations could be measured in the same experiment.

Bubble column reactor experiments

About 75 g of alginate beads, corresponding to 5 g dry wt/l reactor volume, were placed in a bubble column reactor with a working volume of 500 ml. The reactor had an inner diameter of 5.5 cm and was 30 cm high. The supply of oxygen was assured by forcing air through a sintered glass filter in the bot-

tom of the reactor in order to form small gas bubbles. The air flow rate was 1.0 vvm. To ensure that the oxygen concentration was sufficient to fulfill the microorganism's requirements, oxygen was measured with a galvanic oxygen electrode (Johnson et al. 1964) connected to a recorder. The dissolved oxygen level was never below 90% of air saturation.

In order to obtain good mixing properties, the reactor was slightly tapered at the bottom. The reactor was also double walled to facilitate temperature control by circulating water from a water bath. The temperature was maintained at 30°C. The medium consisted of glycerol (0.25 M) in succinate buffer (0.1 M, pH 5.0). Calcium chloride (10 mM) was added in order to stabilize the gel. Medium was fed with a membrane pump (Braun Melsungen FE 211). The volume was kept constant by suction from the surface with a peristaltic pump. The experiments were carried out under sterile conditions.

Solubilization of alginate gel

In order to check the activity of the cells after immobilization, alginate beads were dissolved in 0.1 M citrate buffer (pH 5.0). The buffer was stirred magnetically and dissolution was complete in approximately 30 min. The activity of the cells was then determined as above.

Analysis of dihydroxyacetone

DHA was assayed with a modification of the DNS-method of Miller et al. (1960). In this method DHA is oxidized by dinitrosalicylic acid and a coloured compound is formed. One ml of the sample was mixed with 1 ml of the DNS-reagent. The absorbance was measured at 640 nm by Miller et al., although the absorbance maximum is at much shorter wavelength. The sensitivity of the method was increased in this study by measuring the absorbance at 575 nm. This increased the absorbance by a factor of 3.5. At still shorter wavelengths (470–520 nm) the method is unreliable (Hostettler et al. 1951).

Oxygen analysis by Winkler titration

The method used in this study was a modification of the method in ASTM Standards (American Society for Testing and Materials 1980). The modification was necessary because of the high buffering capacity of the samples. The reagents were the same as in the standard method but the amounts had to be increased. The sample was poured into a bottle with a volume of 116.7 ml. Then 1.4 ml of alkaline potassium iodide solution (150 g/l KI, 700 g/l KOH) and 0.7 ml of manganese sulphate solution (364 g/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$) were added and the stopper was sealed tightly. After completion of the reaction, 2.1 ml of H_2SO_4 (3:1) was added. The bottle was stoppered and shaken until the precipitate was dissolved. The liberated iodine was titrated with a sodium thiosulphate solution.

Results and discussion

Cell immobilization

The immobilization resulted in spherical gel beads. No cells could be detected in the calcium

chloride solution after immobilization as determined by measuring the glycerol oxidizing capacity. In a typical batch 76.8 g of cell-alginate slurry gave 37.9 g of wet catalyst, the shrinkage thus being 51%. The particle diameter was quite uniform, a typical result being 2.20 ± 0.06 mm (mean $\pm$ standard deviation). In order to check if the immobilization procedure harmed the cells, the beads were dissolved in citrate buffer after immobilization and their activity was determined. It was found that their activity was the same as that of cells that had not been immobilized. Additions of citrate and sodium alginate to the medium had no effect on the activity of the cells.

Kinetic experiments

In the kinetic experiments, the rate of glycerol oxidation to DHA by *G. oxydans* cells was measured under different conditions. The oxygen concentration was continuously measured with a polarographic oxygen electrode. Graphs like those in Fig. 1 were obtained. When free cells were used, the reaction rate was independent of the oxygen concentration with the exception of very low concentrations due to the high affinity of the cells for oxygen. Therefore, straight graphs were obtained. When immobilized cells were used, bent graphs were obtained because oxygen transfer was the rate limiting factor.

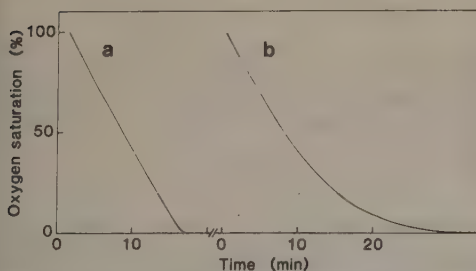


Fig. 1. Oxygen concentration as a function of time as recorded in the kinetic measurements. The oxygen concentration was continuously measured with a polarographic oxygen electrode. The diagram shows one experiment with free cells (a) and one with immobilized cells (b)

From the slope of the curves, the reaction rate could be calculated. In the following experiments, the reaction rate was measured as a function of different parameters.

Reaction rates were recalculated by converting the electrode measurement from partial pressure

of oxygen to a concentration value after standardizing the oxygen concentration with the Winkler-method. The oxygen concentration in the succinate buffer was determined by Winkler titration. The oxygen solubility in 0.1 M sodium succinate buffer (pH 5.0) containing 10 mM CaCl_2 and 0.25 M glycerol, as used in the kinetic experiments, was 91.5% of that in pure water. The determinations were done with air saturated liquids at 30°C . As the concentration of oxygen in pure water saturated with air (1 atm at 30°C) is 7.57 ppm (Montgomery et al. 1964), the concentration in the buffer solution under these conditions was 0.216 mM. If the buffer solution had been saturated with pure oxygen, the solubility would be 1.034 mM.

Influence of temperature

The temperature dependence of the reaction was studied. The optimal temperature for the initial reaction rate was 40°C (Fig. 2). But when choos-

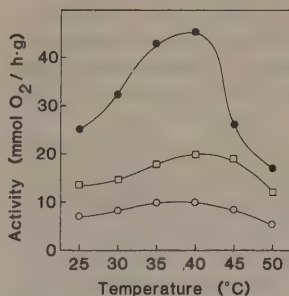


Fig. 2. Temperature dependence of the oxidation of glycerol to DHA by *G. oxydans* cells. The substrate solution was oxygen saturated. The immobilized preparations had the cell densities of 9.6 g/l (□) and 29 g/l (○). The particle diameters were 2.1 mm and 2.3 mm, respectively. The activities for free (●) and immobilized (□, ○) cells are shown in the figure.

ing a suitable temperature for long term operation, the stability of the cells must also be considered. In Fig. 2, the initial reaction rates are given. The remaining activity (measured at 25°C) after 24 h of operation at different temperatures is shown in Fig. 3. The activity of free cells decreased rapidly with increasing temperature. The immobilized cells were more stable. After operation at 25°C , the activity was a little higher than before treatment. Apparently, some activation of the cells or some limited cell growth had taken place. Operation at temperatures above 35°C de-

creased the activity of the immobilized cells considerably. For the rest of the experiments, a temperature of 30°C was chosen.

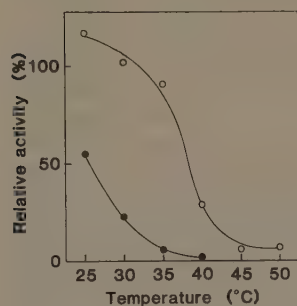


Fig. 3. Temperature stability of *G. oxydans* cells while converting glycerol to DHA. These cells were put in shaker flasks containing 150 ml of 0.50 M glycerol in succinate buffer. The flasks were shaken at different temperatures for 24 h. Then the immobilized cells were recovered by filtration and the free cells by centrifugation (17,000 g, 25 min). The remaining activity of the cells was then measured at 25°C using oxygen saturated media. The activity of untreated cells was set at 100%. Data for free (●) and immobilized (○) cells are shown in the figure

The disappearance of oxygen was measured in all of these measurements. The most important parameter was however, the production of DHA. In order to check if the disappearance of oxygen was accompanied by a corresponding increase in the concentration of DHA, the temperature dependence experiments were repeated and the concentration of DHA was measured. DHA concentration was determined by a modified DNS-method (see *Materials and methods*). The results showed, with respect to the stoichiometry, that the reaction rate measured as the increase of DHA

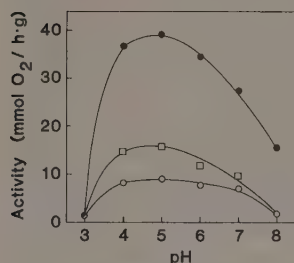


Fig. 4. The pH-dependence of the glycerol oxidation to DHA by *G. oxydans* cells. The following buffers were used; at pH 3, 0.1 M fumarate; at pH 4–6, 0.1 M succinate and at pH 7–8, 0.1 M tris. The immobilized preparations had cell densities of 9.6 g/l (□) and 29 g/l (○). The particle diameters were 2.1 mm and 2.3 mm, respectively. The activities for free (●) and immobilized (□, ○) cells are shown in the diagram

concentration was the same as the rate measured as the decrease in oxygen concentration.

Influence of pH

It was found that a pH of 5 was optimal for the production of DHA by both free and immobilized *G. oxydans* cells (Fig. 4). The pH-optimum curve in this figure was flat when immobilized cells were used. These observations agree well with previous studies of this organism (Holst et al. 1982).

Influence of glycerol concentration

The activity of the cells as a function of the glycerol concentration is presented in Fig. 5. The curves are sigmoidal both for free and immobilized cells, indicating that Michaelis-Menten kinetics are not applicable. The glycerol concentration required to get half the maximal reaction rate was about 5 mM both for free and immobilized cells. In all the remaining experiments, a glycerol concentration of 0.25 M was used unless otherwise stated. At this concentration, glycerol is not a limiting factor.

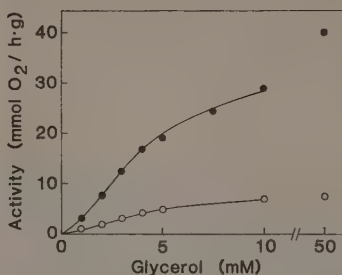


Fig. 5. Productivity of *G. oxydans* cells as a function of the glycerol concentration. The temperature was 30°C. The substrate solutions were saturated with oxygen. Either 10 mg of free cells (●) or 25 mg of immobilized cells (○) were used in each experiment

The influence of oxygen concentration, cell density and particle size

The activity of free cells is independent of the oxygen concentration in a wide concentration range. However, the activity of immobilized cells varied with the oxygen concentration (Fig. 6). Thus, if the production medium is saturated with pure ox-

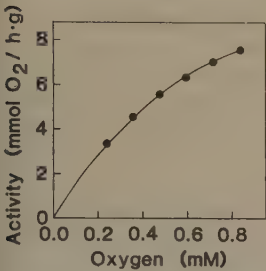


Fig. 6. Productivity of immobilized *G. oxydans* cells as a function of the oxygen concentration in the bulk liquid phase

ygen instead of air the production rate can be greatly increased.

In order to investigate how the particle size and the cell density of the gel influenced the productivity, immobilized preparations with different cell loadings and particle sizes were made. The activity of the cells decreased with increasing particle size, as expected (Fig. 7). Even with the small-

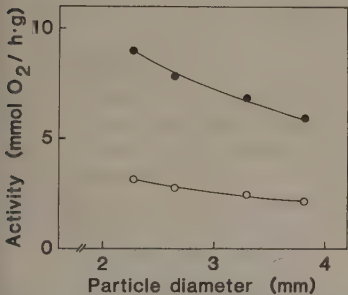


Fig. 7. Productivity of immobilized *G. oxydans* cells as a function of the particle diameter. For each particle diameter the productivities at oxygen concentrations corresponding to saturation with air (O) or oxygen (●) are given

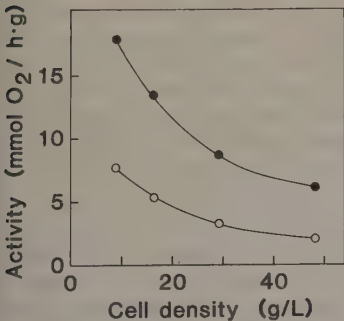


Fig. 8. Productivity of immobilized *G. oxydans* cells as a function of the cell density in the alginate gel. For each cell density, the productivity at an oxygen concentration corresponding to saturation with air (O) or oxygen (●) is shown

lest particle size, the activity was far below that of free cells. In the other set of preparations, the particle size was kept constant and the cell density was varied. As expected, the activity decreased with increasing cell density (Fig. 8).

When immobilized aerobic cells are used, oxygen supply is often a limiting factor (e.g. Tramper et al. 1983). As the transfer of oxygen from the bulk liquid phase to the cells in the matrix is a slow process, the oxygen concentration in the centre of the beads will be low, if not zero, so that only the cells at the surface of the beads will be supplied with enough oxygen to carry out the oxidation reaction at a normal rate. The mean activity of cells in an immobilized preparation will therefore be considerably lower than that of free cells.

The activity of immobilized aerobic cells is dependent on several parameters. The three most important of these are the cells density, the particle size and the bulk concentration of oxygen. If a low cell density, a small particle size and a high bulk concentration of oxygen is used, the activity of free cells can be approached.

A thorough discussion of the importance of the different parameters accompanied by a computer simulation will be published shortly (Adlercreutz, submitted).

Product inhibition

The oxidation of glycerol to DHA is not inhibited to any great extent by the product DHA. Only at a very low glycerol concentration and a very high DHA concentration was a small inhibitory effect noticed (Table 1).

Table 1. Product inhibition of glycerol oxidation to DHA by immobilized *G. oxydans* cells. All substrate solutions were saturated with oxygen

Glycerol (mM)	DHA (mM)	Activity (mmol O ₂ /h·g)
250	0	6.48
250	250	6.42
250	1,000	6.06
10	0	5.94
10	250	5.35
10	1,000	4.72

Operational stability

The operational stability of the immobilized preparation was studied in a continuously operated

bubble column reactor. The reactor was operated for 12 days with a dilution rate of 0.15 h^{-1} giving a residence time of 6.67 h.

The medium used consisted of glycerol (0.25 M) in succinate buffer (pH 5.0, 0.1 M) supplemented with CaCl_2 (0.01 M).

Under these conditions, the half-life of the preparation was found to be 5 days (Fig. 9).

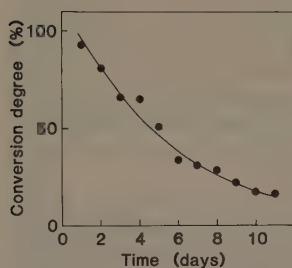


Fig. 9. Operational stability, expressed as conversion degree as a function of time, of *G. oxydans* immobilized in calcium alginate. The residence time was 6.67 h ($D = 0.15 \text{ h}^{-1}$)

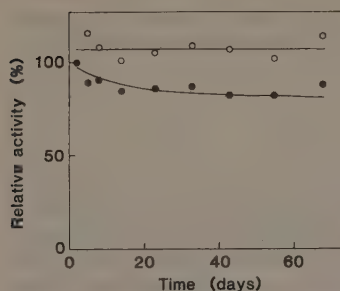


Fig. 10. Storage stability of *G. oxydans* cells. The cells were stored in 0.1 M succinate buffer (pH 5.0) containing 10 mM of CaCl_2 at 4°C for different periods of time. The remaining activity, for free cells (●) and for immobilized cells (○), at an oxygen concentration of 0.90 mM (87% oxygen saturation) is shown

The mechanical stability of the beads was good. No turbidity, measured as optical density at 620 nm, could be detected in the outlet. A slight swelling of the beads was noted. The diameter increased from 2.1 mm to 2.5 mm.

Experiments to investigate the influence of dilution rate (residence time) on the conversion degree were difficult to carry out due to the rather low operational stability. When long residence times were used and a throughput of several reactor volumes was allowed, the decrease in activity made measurements meaningless.

Storage stability

Free and immobilized cells were stored in succinate buffer at 4°C for periods up to 68 days. Under these conditions, the cells were quite stable. A slight decrease in the activity of free cells was observed while no decrease at all could be observed with the immobilized cells (Fig. 10). Storage in a salt solution (0.15 M NaCl, 10 mM CaCl_2) at 4°C gave similar results (data not shown).

Concluding remarks

The present investigation was focused on immobilized resting cells. The reaction studied in this system is well suited for use in the study of a conversion by an immobilized cell preparation. This is because the conversion is a one-step process, the substrate is a small molecule and, finally, that the product is not charged. These facts reduce the environmental influence on the cells in the immobilized state.

A limiting factor, however, is the low solubility of the second substrate, oxygen.

From the studies on the effects of pH and temperature on the immobilized preparations, it is seen in figures 2 and 4, that the profiles are broader than the corresponding curves for the free cells. This may be ascribed to the fact that due to diffusion limitations, an excess of catalytic activity was present under optimal conditions as only the outer layer of the immobilized cells were exposed to the substrate. When the conditions were changed and less substrate was converted by the initially active cells substrate became available to cells placed deeper in the matrix which in turn reduced the observed effect of the changed conditions. In the figures giving the pH-activity profiles as well as that showing the temperature dependence profiles, are seen one curve in each graph obtained, using a preparation of immobilized cells with a reduced cell density. With reduced cell densities the diffusion restrictions found with immobilized cells were less pronounced and thus the appearance of the pH-activity and temperature profiles were closer to those of free cells.

When characterizing a preparation it is easier to use resting cells in comparison to a situation when the cells are growing, since in the latter case leakage of cells from the support to the surrounding medium may take place. This leads to a situation with a population of both immobilized cells and freely suspended cells. Determination of ki-

netic parameters valid for immobilized cells would under such circumstances involve severe experimental problems. However, for actively growing immobilized cells far better operational stability of the immobilized preparation has been demonstrated by, e.g., Seiskari et al. (1984).

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References

Adlercreutz P (submitted) Oxygen supply to immobilized cells: 5. Theoretical calculations and experimental data for the oxidation of glycerol by immobilized *Gluconobacter oxydans* cells with oxygen or p-benzoquinone as the electron acceptor.

Adlercreutz P, Holst O, Mattiasson B (1982) Oxygen supply to immobilized cells: 2. Studies on a coimmobilized algae-bacteria preparation with *in situ* oxygen generation. *Enz Microb Technol* 4: 395–400

Adlercreutz P, Mattiasson B (1982) Oxygen supply to immobilized cells: 3. Oxygen supply by hemoglobin or emulsions of perfluorochemicals. *Eur J Appl Microbiol Biotechnol* 16: 165–170

Adlercreutz P, Mattiasson B (1984) Oxygen supply to immobilized cells: 4. Use of p-benzoquinone as an oxygen substitute. *Appl Microbiol Biotechnol* 20: 296–302

American Society for Testing and Materials (1980) Annual book of ASTM standards, Part 31, D 888, method E, p 538–539

European Federation of Biotechnology (1983) Guidelines for the characterization of immobilized biocatalysts. *Enz Microb Technol* 5: 304–307

Green SR (1967) Dihydroxyacetone. In: Peppler HJ (ed) Microbial technology. p 360, Reinhold Publ Corp, New York

Holst O, Enfors SO, Mattiasson B (1982) Oxygenation of immobilized cells using hydrogen peroxide: A model study of *Gluconobacter oxydans* converting glycerol to dihydroxyacetone. *Eur J Appl Microbiol Biotechnol* 14: 64–68

Hostettler F, Borel E, Deuel H (1951) Über die Reduktion der 3,5-Dinitrosalicylsäure durch Zucker. *Helv Chim Acta* 34: 2132–2139

Johnson MJ, Borkowski J, Engblom C (1964) Steam Sterilizable Probes for Dissolved Oxygen Measurement. *Biotechnol Bioeng* 6: 457–468.

Makhotkina TA, Pomortseva NV, Lomova IE, Nikolaev PI (1981) Glycerol transformation into dihydroxyacetone by polyacrylamide gel immobilized cells of *Gluconobacter oxydans*. *Prikl Biokhim Mikrobiol* 17: 102–106

Miller GL, Blum R, Glennon WE, Burton AL (1960) Measurements of carboxymethylcellulase activity. *Anal Biochem* 2: 127–132

Montgomery HAC, Thom NS, Cockburn A (1964) Determination of dissolved oxygen by the Winkler method and the solubility of oxygen in pure water and seawater. *J Appl Chem* 14: 280–296

Nabe K, Izuo N, Yamada S, Chibata I (1979) Conversion of glycerol to dihydroxyacetone by immobilized whole cells of *Acetobacter xylinum*. *Appl Environ Microb* 38: 1056–1060

Seiskari P, Linko YY, Linko P (1984) Continuous production of gluconic acid by immobilized *Gluconobacter* organisms. Third Eur Congr Biotechnol, abstracts 1: 339–344

Tramper J, Luyben KChAM, van den Tweel WJJ (1983) Kinetic aspects of glucose oxidation by *Gluconobacter oxydans* cells immobilized in calcium alginate. *Eur J Appl Microbiol Biotechnol* 17: 13–18

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Oxygen supply to immobilized cells:

1. Oxygen production by immobilized *Chlorella pyrenoidosa*

Patrick Adlercreutz and Bo Mattiasson

Department of Pure and Applied Biochemistry, Chemical Center, University of Lund, P.O. Box 740, S-220 07 Lund, Sweden

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Oxygen supply at high cell densities of aerobic organisms is a great problem in immobilized cell preparations. To investigate the possibility of in situ oxygen generation the alga Chlorella pyrenoidosa was immobilized on alginate beads. The conditions for optimal oxygen production were investigated and in long term experiments the preparations were successful for at least 30 days.

Keywords: Oxygen production; *Chlorella pyrenoidosa*; oxygen supply; immobilized algae.

Introduction

Most studies with immobilized cells have been performed with anaerobic cells. Transfer of substrate to the cells has often been readily controlled by increasing the substrate concentration in the bulk phase.

Aerobic cells need oxygen as well as the substrate to be converted. This creates problems, long known in conventional fermentation technology.

Under atmospheric pressure at room temperature oxygen from the atmosphere is soluble in water to ~ 0.25 mM. This means that bioconversion is often limited by oxygen deficiency. Efforts have been made to reduce the hampering effect of the low solubility of oxygen. The use of pure oxygen improves the oxygen supply.¹ But these measures have often not solved the problems and other methods have been tried.

When studying one oxidase-catalysed reaction, steroid transformation by *Nocardia*, Lilly and coworkers found that improved productivity was obtained if an organic solvent was used instead of buffer.² The effect was ascribed to both better solubility of the steroid and a higher oxygen tension in the solution. Thus, a change of incubation medium to an organic phase could improve the reaction conditions provided the cells tolerate the new environment.

Oxidases often generate hydrogen peroxide, which may *per se* be deleterious to the cells, but the hydrogen peroxide can be degraded using catalase [hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6] which leads to the release of some oxygen, resulting in a slightly better oxygen supply. In classical fermentation technology stirring is widely used for improving oxygen transfer. Since diffusion in immobilized enzyme and cell preparations is restricted, only slight effects of stirring may be attained, besides which any such effect will be counteracted by attrition of the supporting material. Furthermore, the limited solubility of oxygen in water makes it very difficult to supply such a dense cell population as an immobilized preparation with

enough oxygen from external sources. The solution to this dilemma would then be either to operate the process batch-wise or to work with much higher dilutions of the substrate — some 1000 times higher than hitherto used — and instead operate with very high dilution rates. This is not a realistic situation since the product stream would be too dilute and the reactor would not tolerate the flows that would be needed. Other solutions have therefore been tried, e.g. generation of oxygen *in situ*.

In analytical applications where samples were analysed intermittently, coimmobilization of red blood cells (RBC) and oxidases provided an analytical unit with improved operational characteristics because of an improved oxygen supply from the haemoglobin in the cells.³ Another way to enhance the oxygen supply locally in an analytical system was tried: a pO_2 electrode with glucose oxidase [β -D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4] was equipped with a Pt-electrolysis unit to generate oxygen locally.⁴

Other biochemical methods have been used to supply oxygen, such as coimmobilizing catalase with the oxygen consuming unit so that oxygen is generated by catalase catalysed decomposition of hydrogen peroxide and then consumed within the same unit. This approach was first tested with *Gluconobacter oxydans* an organism with a high natural catalase content.⁵ The dominating method in nature to generate oxygen is photosynthesis. The present paper concerns the use of immobilized algae for oxygen production with the intention of coimmobilizing them with an oxygen-consumer.

Experimental

Organism and cultivation

Chlorella pyrenoidosa (No. 252 in the Collection of Algae at Indiana University)⁶ was grown in Erlenmeyer flasks in a medium containing per litre distilled water: 2.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 1.25 g KNO_3 ; 1.25 g KH_2PO_4 ; 165 mg $\text{Na}_3\text{citrate} \cdot 2\text{H}_2\text{O}$; 111 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 4.0 mg $\text{Fe}_2(\text{SO}_4)_3$

Part of this work was presented at the IVA-seminar on Biotechnology, Lund, 27 January 1982.

• $x\text{H}_2\text{O}$; 2.9 mg H_3BO_3 ; 1.8 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$; 0.2 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 0.05 mg CuSO_4 and 0.02 mg MnO_3 . Sterile air was bubbled through the cultures. The flasks were illuminated with 4 fluorescent lamps (LUMA 20 W warm white). After cultivation for 2–3 weeks the algae were harvested by centrifugation (10 000g, 15 min).

Quantification of algae

The amount of algae was determined as cell mass (dry weight). A cell suspension was filtered through a membrane filter (Sartorius, 0.45 μm). The filter was then washed with distilled water and dried at 105°C for 75 min.

Immobilization

A suspension of cells in the culture medium was mixed with an equal volume of 4% (w/w) sodium alginate (KEBO, Malmö, Sweden). The mixture was dropped from a hypodermic needle (0.9 mm I.D.) into a solution of 0.1 M CaCl_2 . Beads of calcium alginate gel, in which the cells were entrapped, formed immediately. The CaCl_2 solution was gently stirred for 2 h in order to stabilize the beads. The beads were then collected by filtration and kept in a solution containing 20 mM NaCl and 10 mM CaCl_2 until use.

Solubilization of gel beads

Gel beads were put in a 0.2 M solution of trisodium citrate (0.5 ml per bead) in a test tube. The test tubes were shaken intermittently. After about 1 h all the calcium alginate matrix had completely dissolved. The number of cells in the beads could then be counted. The beads did not dissolve so well in lower and higher concentrations of sodium citrate.

Experimental set-up

The gel beads containing immobilized cells were packed in a glass column (0.55 $\times$ 8.5 cm). The small reactor was illuminated with a 1000 W lamp (Osram SLG 1000 with a 1000 W halogen lamp). To prevent the column temperature from rising above room temperature, a heat trap containing a copper sulphate solution was placed between the lamp and the column. The light passed through 60 mm of a 1.1% (w/v) aqueous solution of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. The solution was cooled with tap water. A buffer solution was pumped through the column by a peristaltic pump (LKB). The concentration of dissolved oxygen in the effluent from the column was measured with a polarographic oxygen electrode (Beckman) placed in a 5 ml cell, in which the solution was stirred magnetically. The connection between the column and the oxygen electrode was made of stainless steel to prevent leakage of oxygen. This experimental set-up was used throughout except in the long term experiment (see below).

Optimizing experiments

General remarks

All media contained 10 mM CaCl_2 to stabilize the beads. Carbonate and nitrate were used as substrates for algal oxygen production. Carbonate was added as NaHCO_3 and nitrate as NaNO_3 . Some different buffers were used in different pH-intervals (sodium fumarate, maleic acid, Tris). Immediately before each experiment the pH values of the media were adjusted with aqueous NaOH or HCl.

All optimizing experiments were made with a flow rate of 1.24 ml min⁻¹ and with a cell density of ~4 mg dry weight/ml reactor volume (except when the cell density was varied). All experiments were carried out at room temperature.

Variation of pH

Media with different pH values were pumped through the column. Each experiment was continued until the concentration of dissolved oxygen in the effluent from the column reached a constant value. The light intensity was 7.4 klx throughout the experiment. In one series of experiments the media contained no substrate, only CaCl_2 and buffer substances (10 mM fumarate–HCl for pH 4–6 and 10 mM Tris–HCl for pH 7–8). The same media supplemented with 10 mM nitrate were used in another series. These two experimental series (except the experiments at pH 4) were performed on one day with the same beads. The experiments at pH 4 were done with another portion of beads. In still another series the media contained 10 mM carbonate and 10 mM CaCl_2 . The same media supplemented with 10 mM nitrate were then used to investigate the effect of the simultaneous exposure to both substrates. These experiments were performed on one day with the same beads. Each value is the mean of at least two separate determinations.

Variation of carbonate concentration

The column was illuminated with 13.8 klx. Media containing 10 mM Tris, 10 mM CaCl_2 and varying concentrations of carbonate (0–20 mM, pH 7.0) were pumped through the column until the concentration of dissolved oxygen in the effluent reached a constant value. The carbonate concentration was then raised to the next value. The same beads were used throughout the experiment.

Variation of light intensity

Four series of runs were made. All the media contained 10 mM CaCl_2 and had a pH value of 7.0. In one series the medium contained 10 mM Tris, in another 10 mM Tris and 10 mM nitrate, in still another 10 mM carbonate and in a final series 10 mM carbonate and 10 mM nitrate. The light intensity was varied within a range of 2.7–33.5 klx (the distance between the lamp and the column was changed, and the light intensity was measured with a lux meter). The same beads were used throughout the experiment. The experiments with media containing carbonate were performed on one day and the other series on the next day.

Variation of cell density

To investigate the effect of cell density on the oxygen productivity, seven batches of alginate beads containing algae were prepared (between 0.5 and 62 mg dry weight of algae per ml reactor volume). A medium containing 10 mM carbonate, 10 mM Tris and 10 mM CaCl_2 (pH 7.0) was pumped through the column. The column was illuminated with 7.4 klx until the concentration of dissolved oxygen in the effluent reached a constant value (about 90 min). The light intensity was then increased to 20.5 klx. After about 40 min the oxygen concentration had stabilized and the experiment was terminated.

Testing long term oxygen production

In this 30 day experiment the type of column used was the same as that used in the optimizing experiments. In the beginning the column contained 7 mg dry weight *Chlorella* cells. The column was placed between two 20 W fluorescent lamps (see Organism and cultivation). The distance between the column and each lamp was 45 mm. The fluorescent lamps produced much less heat than the 1000 W lamp used

in the optimizing experiments and so the heat-trap was omitted.

A medium containing 10 mM carbonate, 10 mM maleate and 10 mM CaCl_2 (pH 6.0) was pumped through the column at a rate of 0.65 ml min^{-1} . During three periods (day 5–13, 15–16, 19–20) the medium was also supplemented with 1 mM nitrate.

In order to reactivate the algae, the medium was replaced by the culture medium (see Organism and cultivation) for short periods (30–40 min).

The concentration of dissolved oxygen in the effluent from the column was measured continuously for 30 days.

Results and discussion

The present investigation was performed to find optimal conditions for oxygen production by immobilized *Chlorella pyrenoidosa* cells.

Oxygen production as a function of pH

Carbonate is apparently a more efficient substrate than nitrate at pH values between 5 and 8 (Figure 1). Nitrate plus carbonate is a little better than carbonate alone. These results are in agreement with those reported for *Chlorella vulgaris*⁷ and *Chlorella fusca* var. *vacuolata*.⁸ The response to nitrate is known to be very dependent on the cultivation conditions.⁸

During photosynthesis electrons are transported from an electron donor, usually water, to reduce NADP^+ . Electrons flow from NADPH to electron acceptors. The most common electron acceptor is carbon dioxide and the products in this case are carbohydrates. However, many plants can also use nitrate as electron acceptor. The products are reduced nitrogen compounds, such as ammonia. Our experiments indicate that *Chlorella pyrenoidosa* can use both carbon

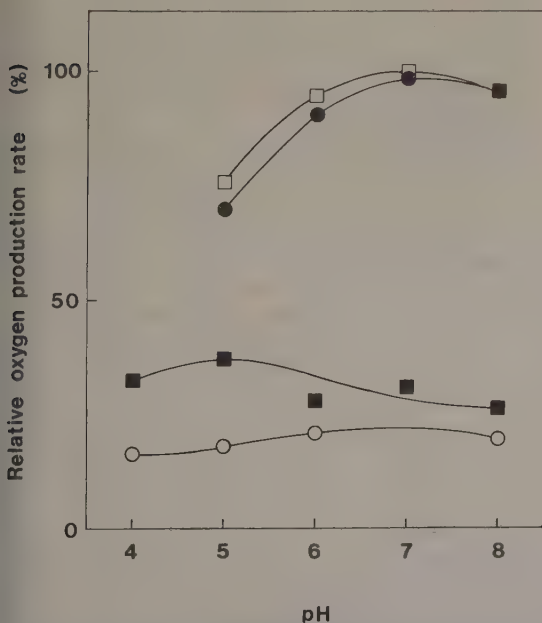


Figure 1 Effect of pH on oxygen production rate of immobilized *Chlorella pyrenoidosa* without substrate (○), with nitrate (■), with carbonate (●) and with carbonate plus nitrate (□). Maximal productivity was set at 100%

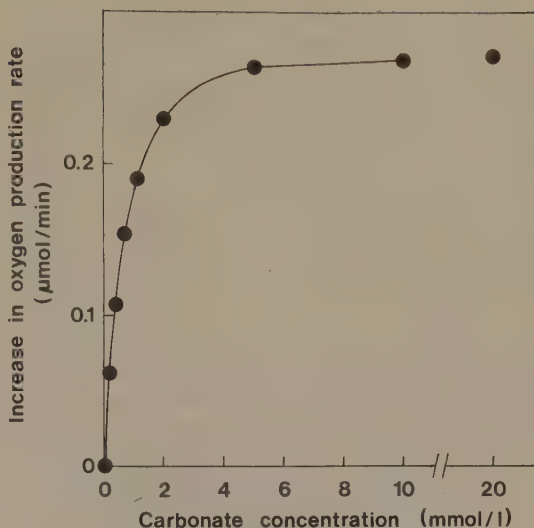


Figure 2 Increase in oxygen production rate of immobilized *Chlorella pyrenoidosa* due to addition of carbonate to the medium. The oxygen production rate without carbonate was $0.084 \mu\text{mol min}^{-1}$

dioxide and nitrate as electron acceptors, and that carbon dioxide is the main electron acceptor.

Maximal oxygen production rate was obtained at pH 7. However, in the systems studied pH values above 6 are not recommended in lengthy experiments with carbonate as substrate, since the solutions used are then supersaturated with respect to calcium carbonate. Initially no negative effects of the supersaturated solutions are observed, but later calcium carbonate precipitates appear within the reactor system. With another matrix, which does not require calcium ions, the optimal pH 7 could be used. Furthermore, at low pH values other problems arise. Because of the evolution of carbon dioxide the stability of carbonate solutions at low pH values is poor. In the rest of the optimizing experiments pH 7 was used, while pH 6 was used in the long term experiment.

Oxygen production as a function of carbonate concentration

When the oxygen production rate of a preparation of immobilized *Chlorella pyrenoidosa* cells was plotted against the carbonate concentration, the curve obtained was hyperbolic (Figure 2). The oxygen production rate was fairly constant at carbonate concentrations above 5 mM. In the rest of the optimizing experiments a carbonate concentration of 10 mM was used.

Influence of light intensity on oxygen production

When a buffer solution without nitrate and carbonate was used as medium for *Chlorella pyrenoidosa* cells, saturation was reached at a light intensity below 3 klx. With nitrate or carbonate in the medium the oxygen production rate increased with the light intensity. The oxygen production rate did not reach its maximum in the range tested ($\leq 33.5 \text{ klx}$) though some levelling off was observed (Figure 3). Here, as in previous experiments, carbonate proved to be a better substrate than nitrate, and the highest oxygen production rates were obtained with both carbonate and nitrate in the medium.

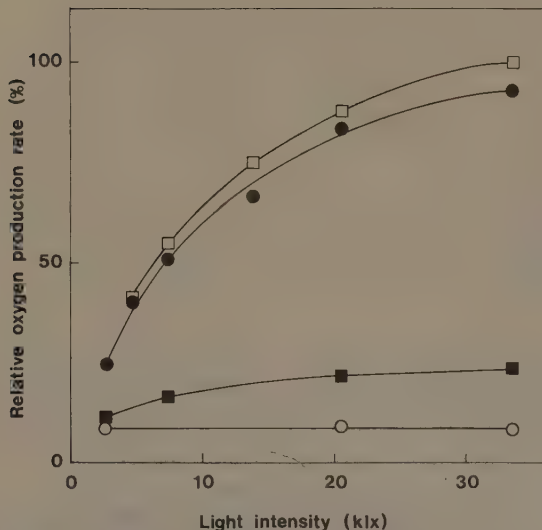


Figure 3 Effect of light intensity on oxygen production rate of immobilized *Chlorella pyrenoidosa* without substrate (○), with nitrate (■), with carbonate (●) and with carbonate plus nitrate (□). Maximal productivity was set at 100%

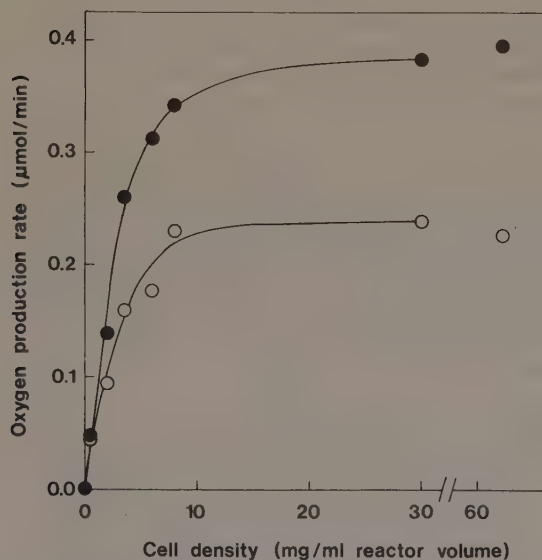


Figure 4 Effect of cell density on oxygen production rate of immobilized *Chlorella pyrenoidosa* at 7.4 klx (○) and 20.5 klx (●)

Oxygen production rate as a function of cell density in the immobilized preparation

At low cell densities the oxygen production rate varied approximately with the dry weight of algae in the column (Figure 4). However, when more algae were used not all the cells were reached by the light and the efficiency decreased. No obvious increase in oxygen production rate occurred at cell densities exceeding 10 mg ml^{-1} reactor volume. The tests were performed at two different light intensities: at 20.5 klx the oxygen production rate was higher than at 7.4 klx, even at low cell densities.

It appears that reactor design is extremely important in light dependent reactions, but such considerations are outside the scope of this investigation.

Long term oxygen production

In an effort to evaluate the long term stability of the oxygen production by immobilized *Chlorella pyrenoidosa* cells, a 30 day incubation was tried. During the first four days carbonate only was added to the buffer. The oxygen production rate slowly decreased. Addition of nitrate to the medium had no effect on the productivity.

In an effort to see whether the algae could be reactivated while immobilized, the beads were treated with the culture medium on the 8th day of the experiment. This medium contained phosphate, magnesium and other ions that impair the stability of the alginate and eventually dissolve the beads. The treatment with the culture medium should thus not be too long. Periods of 30–40 min were used. The algae were reactivated in this way at intervals of 1–3 days. This treatment kept the beads fairly intact during the experiment. The oxygen production rate increased to approximately its initial value, and remained at this level throughout the experiment.

Some decrease in the observed oxygen production rate was caused by infecting oxygen consuming organisms in the reactor, but these could easily be removed by washing the beads. Such washings with a solution containing 10 mM CaCl_2 and 20 mM NaCl were performed five times during the experiment.

The oxygen production during the last 24 h (the 30th day) of the experiment was 93% of that during the first 24 h.

During the first seven days the immobilized algae multiplied rather slowly (Figure 5). Addition of nitrate to the medium did not cause the algae to multiply faster. The reactivation caused rapid growth inside the beads. The growth ceased after a few days. The scatter in Figure 5 was due to the fact that samples taken during the experiment consisted of only two beads. Ten beads were used for the final determination (after 30 days), which is thus more accurate. Besides the growth inside the beads, algae also leaked from the column.

The oxygen production rate did not increase significantly when the number of algal cells in the column increased.

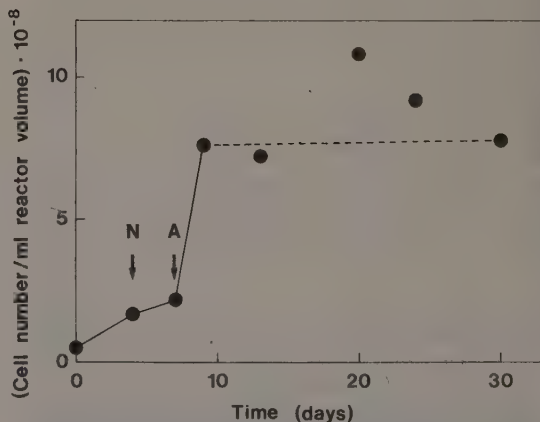


Figure 5 Number of algal cells per ml reactor volume as a function of time during continuous oxygen production. N, addition of nitrate to the medium; A, the first reactivation

This was due, at least partly, to the effect of saturation at high cell densities (Figure 4). During almost the whole experiment light was the limiting factor, which was even more obvious when the cell density in the beads increased on reactivation.

General discussion

Algae have hitherto attracted relatively little attention in the biotechnological field dealing with immobilized biochemical species. Most efforts have so far been focused on the production of reduction equivalents and ultimately hydrogen.^{9,10} In such studies oxygen generation has been a severe disturbance that should, if possible, be eliminated.¹¹ Algae possess the metabolic capacity to synthesize a large number of substances,¹² but so far very little has been done to exploit this commercially.

When photosynthetic reactions have been used to generate a certain product, gas formation is a serious technical problem. This is particularly so when dealing with immobilized cells since gas formation within the gel lattice hampers the mass transfer and thus reduces the metabolic capacity of the preparation.

In this study the possibilities of using immobilized *Chlorella pyrenoidosa* as an oxygen producer were investigated. It was found that the algae tolerated the immobilization procedure very well, and in the presence of carbonate produced oxygen at a fairly constant rate for one month. The goal is to coimmobilize the algae together with an oxygen-consuming organism, which requires oxygen to carry out a certain reaction. This would provide an effective biocatalyst with a long life span.

Using photosynthetic mechanisms for oxygen production places very specific demands on reactor design in order to achieve high efficiency in the light harvesting procedure. Those demands have already been under investigation in other light-harvesting projects^{13,14} and thus much work has already been done. The process could easily be regulated by varying the light intensity or the flow rate of the medium. Problems might of course occur. Some organisms are known to produce substances which are poisonous to other organisms. Possible toxic effects ought to be evaluated before the organisms are paired. In the present case it should be possible to find a suitable alga for most oxygen-consuming organisms.

Another possible problem is that the alga might metabolize the substrate intended for the oxygen consuming organism or the product of the organism. This problem is rather likely to occur in some carbohydrate transformations.

There are also possible positive interactions. The oxygen-consuming organism can, for example, produce carbon dioxide, which can be utilized by the alga. This is an example of recirculation within the system where organisms create a favourable environment for each other. Many such systems, where two organisms live together to the benefit of both, occur naturally. Such organisms are known as symbionts and the condition as symbiosis. Algae are known to participate in many symbiotic relationships. The best studied such system is that of lichens, where an alga and a fungus together make up the lichen thallus, in which the cell species supplement each other's metabolic capacity.

In spite of the large number of potential 'lichen-combinations' it is probable that only a minor portion actually exist. This may be due to different factors, e.g. production of toxic substances. The immobilization technique enables the biotechnologist to produce synthetic symbionts and thus produce combinations that for one reason or the other do not normally occur.

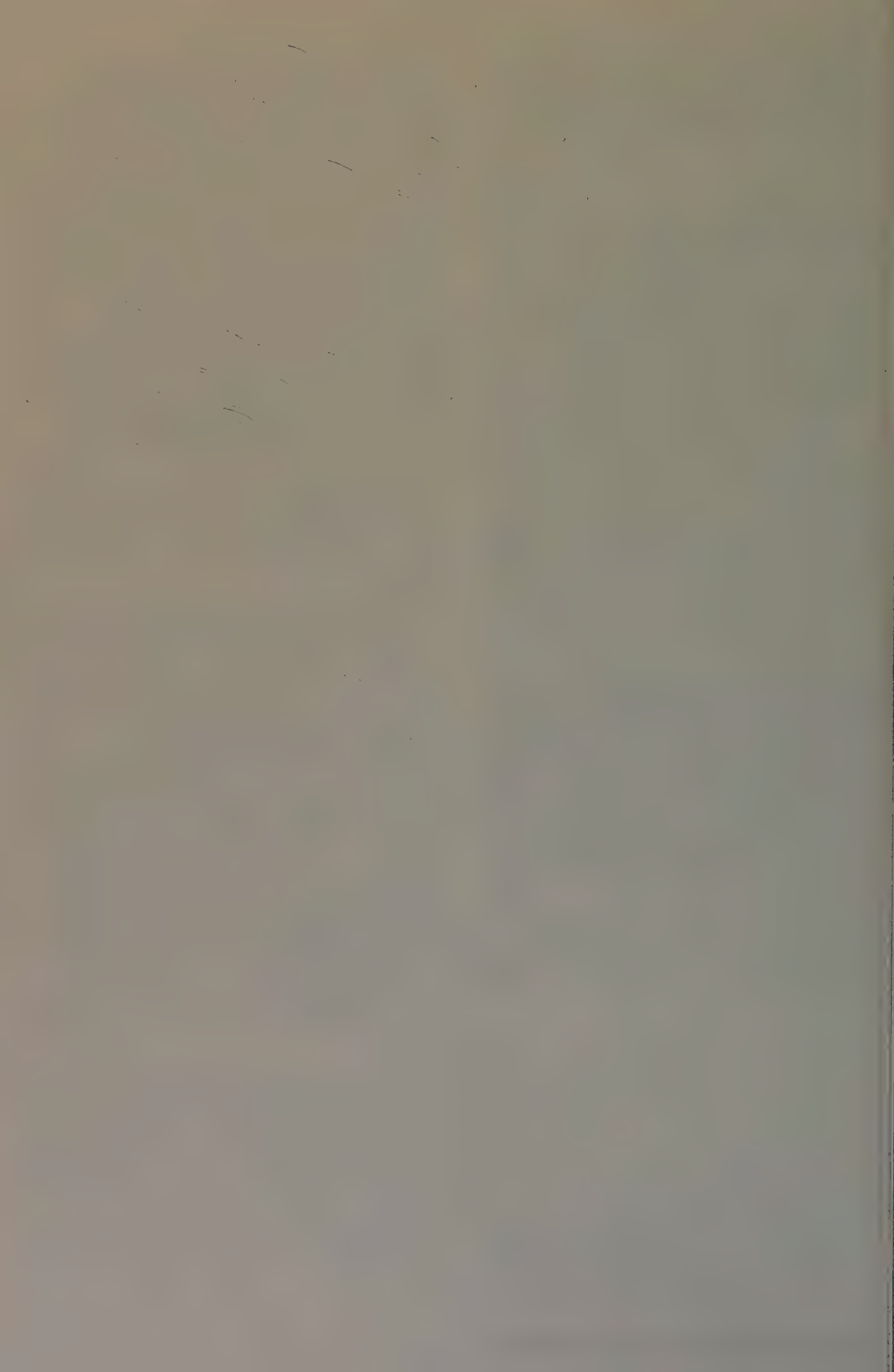
Coimmobilization of *Chlorella pyrenoidosa* and an oxygen consuming bacteria, *Gluconobacter oxydans*, is discussed in a future paper.

Acknowledgement

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References

- 1 Makhotkina, T. A. Pomortseva, N. V., Lomova, I. E. and Nikolaev, P. I. *Prikl. Biokhim. Mikrobiol.* 1981, 17, 102–106
- 2 Buckland, B. C., Dunnill, P. and Lilly, M. D. *Biotechnol. Bioeng.* 1975, 17, 815–826
- 3 Mattiasson, B. and Borrebaeck, C. *FEBS Lett.* 1978, 85, 119–123
- 4 Enfors, S.-O. *Enzyme Microb. Technol.* 1981, 3, 29–32
- 5 Holst, O., Enfors, S.-O. and Mattiasson, B. *Eur. J. Appl. Microbiol. Biotechnol.* 1982, 14, 64–68
- 6 Starr, R. C. *Am. J. Bot.* 1964, 51 (9), 1013–1044
- 7 Gerster, R. *Plant Sci. Lett.* 1975, 5, 255–260
- 8 Thomas, R. J., Hipkin, C. R. and Syrett, P. J. *Planta* 1976, 133, 9–13
- 9 Kayano, H., Matsunaga, T., Karube, I. and Suzuki, S. *Biochim. Biophys. Acta* 1981, 638, 80–85
- 10 Kayano, H., Karube, I., Matsunaga, T., Suzuki, S. and Nakayama, O. *Eur. J. Appl. Microbiol. Biotechnol.* 1981, 12, 1–5
- 11 Benemann, J. R., Berenson, J. A., Kaplan, N. O. and Kamen, M. D. *Proc. Natl. Acad. Sci. USA* 1973, 70, 2317–2320
- 12 Proc. Xth International Seaweed Symposium (Tore Levring, ed.) Walter de Gruyter, Berlin, New York, 1981
- 13 Lee, Y. K. and Pirt, J. J. *Gen. Microbiol.* 1981, 124, 43–52
- 14 GBF Wissenschaftlicher Ergebnisbericht 1978, pp. 9–10



Oxygen supply to immobilized cells:

2. Studies on a coimmobilized algae–bacteria preparation with *in situ* oxygen generation

Patrick Adlercreutz,* Olle Holst† and Bo Mattiasson*

*Department of Pure and Applied Biochemistry, and †Department of Technical Microbiology, Chemical Center, University of Lund, P.O. Box 740, S-220 07 Lund, Sweden

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A coimmobilized mixed culture of algae, Chlorella pyrenoidosa, and bacteria, Gluconobacter oxydans, has been studied. The conversion of glycerol to dihydroxyacetone (1,3-dihydroxy-2-propanone), catalysed by the bacteria, was used to indicate the oxygen supply in the immobilized preparation. The oxygen produced by the algae in the coimmobilized preparation was used by the bacteria more effectively than when the cells were immobilized separately and mixed within the reactor. A preparation consisting of only bacteria and no algae was much less effective. The coimmobilized preparation was used in the continuous production of dihydroxyacetone for six days without any significant loss of activity.

Keywords: Symbionts; oxygenation; immobilized *Chlorella*; immobilized *Gluconobacter*; coimmobilization

Introduction

Oxygen supply to dense cell populations is a troublesome problem in conventional fermentation technology, and much work has already been done to get rid of this bottleneck. Immobilization of cells makes it possible to operate with even higher cell densities than those conventionally used in fermentation processes. Thus, the limitations due to insufficient oxygen supply should be stressed even more in this case. Yet very little has so far been done to improve this unsatisfactory situation. If no new technological methods are developed to solve these problems, several restrictions (such as particle size and cell density) will be encountered when designing an immobilized aerobic cell preparation. Reduced particle size will promote supply of substrates to the interior of the particle and so will a reduction in cell density. On the other hand, some advantages of using immobilized cell preparations, the high catalytic density and the ease of handling of these preparations, will be severely reduced.

Oxygen can be supplied from an external source or be generated *in situ*. A great advantage of the latter method is that mass transfer problems may be markedly reduced. A previous paper reported the use of hydrogen peroxide as an oxygen source.¹ The use of an immobilized preparation containing the oxygen consumer with high catalase [hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6] activity to liberate oxygen from the peroxide

increased the oxidative capacity substantially as compared to when oxygen was supplied with the buffer only.

Another biological approach in the generation of oxygen is the photolysis of water. A preceding paper reported the use of immobilized *Chlorella pyrenoidosa* cells for generating oxygen *in situ*.²

Coimmobilization involves certain advantages over free solution or immobilization in separate particles.^{3,4} Thus it has been shown that, as far as enzymes are concerned, a higher overall rate can be expected when one of the reaction steps is thermodynamically unfavourable, and when both are favourable, the lag period is substantially shorter. Furthermore, pH optima for sequences of catalytic reactions may be different when immobilized.⁵ The present report deals with the coimmobilization of the oxygen generator, *Chlorella pyrenoidosa* and the consumer *Gluconobacter oxydans*, where the latter is used as a general model of an oxygen consumer. *G. oxydans* catalyses the reaction:

glycerol -----> dihydroxyacetone

where oxygen is an acceptor of the hydrogen eliminated.

Experimental

Organisms and cultivation

Chlorella pyrenoidosa (No. 252 in the Collection of Algae at Indiana University)⁶ was grown as described earlier.² *Gluconobacter oxydans* (ATCC 621) was cultivated as previously described.¹ Quantification of cells was done by dry weight determinations.^{1,2}

Immobilization

Cells were immobilized in calcium alginate gel as described earlier.² The beads were stored in the dark in buffer solutions containing 10 mM calcium chloride at 4°C until use.

Experimental set-up

The experimental set-up used here consisted of a continuous flow system with a small column reactor (0.55 × 8.5 cm) and is described in detail in the previous paper.² The concentration of dihydroxyacetone was measured instead of the oxygen concentration in the effluent from the column. A 1000 W lamp (Osram SLG 1000) was used as a light source in the optimizing experiments, while fluorescent lamps (LUMA warm white, 4 × 20 W) were used in the long term experiments.

Identification and quantification of dihydroxyacetone

Dihydroxyacetone was identified on high performance thin layer chromatography (h.p.t.l.c.) plates as previously described.¹ Dihydroxyacetone was assayed as a reducing substance using the DNS method of Miller *et al.*⁷

Bio-assay for toxic byproducts of the algae

The following bio-assay was used to ascertain whether the algae *C. pyrenoidosa* produced any toxic byproducts capable of interfering with *G. oxydans*.

A suspension of *G. oxydans* was spread on a Petri dish containing glycerol-agar. The composition of the agar has been described earlier.¹ Filter paper discs with a diameter of 10 mm were placed on the agar and to them was added the medium in which the algae had been cultured, in one set of tests with and in another without algal cells. The tests were run in two parallel sets where one was first incubated in the light (~20 klx) for 2 h directly after application of the incubated pieces of paper to the agar plates. The plates were afterwards incubated in the dark for 46 h at 28°C. Immediately after application of the pieces of paper the other set of plates was placed in the dark incubator for 48 h.

Optimizing experiments

General remarks

Unless otherwise stated, all media contained 10 mM CaCl₂ to stabilize the beads and 30 mM glycerol as a substrate for *Gluconobacter oxydans*. Carbonate (NaHCO₃) was used as a substrate for algal oxygen production. Some different buffer substances were used in different pH intervals (sodium fumarate, maleic acid, succinic acid, Tris). The pH values of the media were adjusted with aqueous NaOH or HCl immediately before use. Unless otherwise stated, the optimizing experiments were made with a flow rate of 0.20 ml min⁻¹.

Preliminary experiments showed that the concentration of dihydroxyacetone in the effluents from the columns became fairly constant after 1–1.5 h. In the optimizing experiments the samples were collected from 1 h 40 min to 2 h after the start of each run. All experiments were carried out at room temperature.

Immobilized preparations used

Three columns (vol. 2 ml), each containing 12 mg (dry weight) immobilized *Gluconobacter oxydans*, were prepared. One of the columns contained *Gluconobacter* only, while the other two columns contained 10 mg (dry weight)

Chlorella pyrenoidosa also. In one of the two columns half of the beads contained bacteria; the other half algae. The two immobilized cell preparations were mixed in the reactor. In the third column the algae and the bacteria were mixed before immobilization, i.e. all beads contained both algae and bacteria.

A medium containing 30 mM carbonate, 30 mM glycerol, 30 mM maleate and 10 mM CaCl₂ (pH 6.0) was pumped through the columns at a flow rate of 0.10 ml min⁻¹. The illumination was 13.8 klx. The rate of dihydroxyacetone production was determined.

Studies on the pH-dependence of the system

Cells of *Gluconobacter oxydans* and *Chlorella pyrenoidosa* were mixed and then immobilized in calcium alginate gel. Columns containing 12 mg (dry weight) bacteria and 17 mg (dry weight) algae per ml reactor volume were prepared. The rate of dihydroxyacetone production in media of varying pH was determined. At each pH value one medium without carbonate and one medium with 30 mM carbonate were tested. The buffers used were 0.1 M fumarate-HCl (pH 4–5), 0.1 M maleic acid-NaOH (pH 6) and 0.1 M Tris-HCl (pH 7–8). The light intensity used was 20.5 klx.

Influence of carbonate concentration on the performance of the whole system

Columns containing the same amounts of cells as in the previous experiment were used. Media containing 30 mM glycerol, 100 mM maleate, 10 mM CaCl₂ and varying concentrations of carbonate (0–100 mM, pH 6.0) were pumped through the columns. The light intensity used was 20.5 klx.

Influence of light intensity

Columns containing the same amounts of cells as in the previous experiments were used. The medium contained 30 mM carbonate, 30 mM glycerol, 100 mM maleate and 10 mM CaCl₂ (pH 6.0). The light intensity was varied between 2.9 and 33.5 klx.

Variation in amount of bacteria

Columns (vol. 2 ml) containing 34 mg (dry weight) algae coimmobilized with varying amounts of bacteria were prepared. A medium containing 30 mM carbonate, 30 mM glycerol, 100 mM maleate and 10 mM CaCl₂ (pH 6.0) was pumped through the columns. The light intensity used was 20.5 klx.

Variation of the amount of algae at constant amount of bacteria

Columns (vol. 2 ml) containing 24 mg bacteria (dry weight) coimmobilized with varying amounts of algae were prepared. A medium containing 30 mM carbonate, 30 mM glycerol, 100 mM maleate and 10 mM CaCl₂ (pH 6.0) was pumped through the columns. The light intensity used was 20.5 klx.

Storage stability

Chlorella pyrenoidosa and *Gluconobacter oxydans* immobilized in calcium alginate were stored in two different buffers, maleate or succinate based, at 4°C in the dark for two weeks. Maleate-based buffer contained 100 mM maleate and 10 mM CaCl₂ (pH 6.0), while succinate based buffer contained 100 mM succinate and 10 mM CaCl₂ and had a pH of 5.0. Samples containing 13 mg algae

and 18 mg bacteria were taken. The samples were packed in columns (vol. 2 ml) and maleate-based buffer supplemented with 30 mM carbonate and 30 mM glycerol was pumped through the columns. The light intensity used was 13.8 klx.

Testing the long-term stability of the coimmobilized system

Two columns (vol. 2 ml) were prepared. One column containing 25 mg (dry weight) immobilized *Gluconobacter oxydans* was used as a blank. The other column also contained 34 mg (dry weight) *Chlorella pyrenoidosa* coimmobilized with the bacteria.

The columns were illuminated by four fluorescent lamps (LUMA 20 W warm white). The distance between the columns and each lamp was 45 mm. A medium containing 5% (v/v) of the culture medium of the algae, 30 mM carbonate, 30 mM glycerol, 100 mM succinate and 20 mM CaCl_2 (pH 5.0) was pumped through the columns at a rate of 0.20 ml min^{-1} . Samples were taken from the effluents of the columns at regular intervals for 6 days.

Results and discussion

Effect of algal products on the bacteria

The bio-assay described above was used to investigate the effect of products of *Chlorella pyrenoidosa* on *Gluconobacter oxydans*. Bacterial growth in the Petri dishes showed no inhibition zones. These results show that, under the conditions used, *Chlorella pyrenoidosa* does not produce any substance that impairs the growth of *Gluconobacter oxydans*.

Effect of coimmobilization

To find out whether the use of algae for oxygen production within the same reactor as an immobilized oxygen consuming organism offered any advantages, three different columns were prepared. One column was prepared with bacteria only (blank); one, with bacteria in half the gel and algae in the other half, with the two preparations mixed in

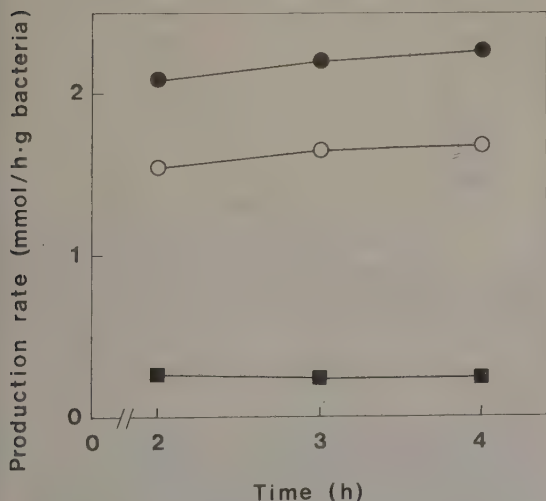


Figure 1 Rate of dihydroxyacetone production of three immobilized cell reactors. One reactor contained *Gluconobacter oxydans* only (■), while the others contained *Chlorella pyrenoidosa* also. The algae were immobilized in separate beads (○) or coimmobilized with the bacteria (●)

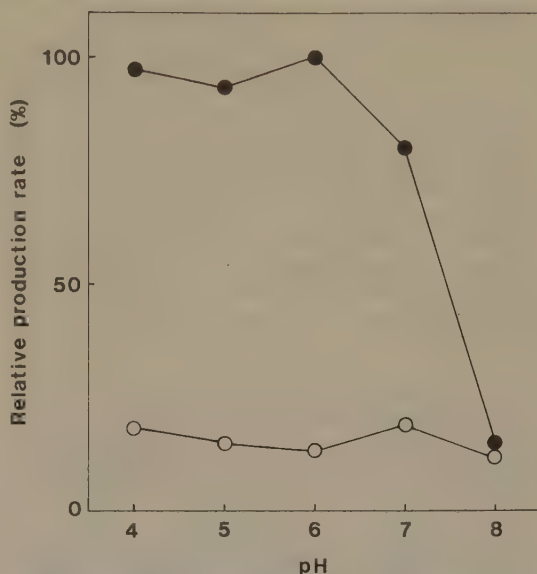


Figure 2 Effect of pH on dihydroxyacetone production rate of coimmobilized *Chlorella pyrenoidosa* and *Gluconobacter oxydans* without carbonate (○) and with 30 mM carbonate (●). Maximal productivity was set at 100%

the reactor; and the third, with gel with coimmobilized algae and bacteria. The column without algae was far less effective than the columns with algae (Figure 1). The column where all the beads contained both algae and bacteria was more effective than the column where some beads contained algae and others, bacteria. This difference is due partly to better utilization of the light when the algae are more widely spread and partly to a more favourable micro-environment. The proximity between the oxygen producer and the oxygen consumer within the beads with coimmobilized cells results in the generation of a favourable micro-environment, where the enhanced oxygen tension around the algae is positively influencing the metabolism of the bacteria. In the rest of the experiments the cells were therefore always mixed before immobilization.

pH-dependence

When no carbonate was used, the rate of dihydroxyacetone production was fairly constant in the pH range 4–8 (Figure 2). A broad optimum around pH 7 was noted. When carbonate was used as substrate for the algae the dihydroxyacetone production rate was rather constant between pH 4 and 6, but it decreased at pH 7 and at pH 8 it was almost as low as without carbonate. This decrease in productivity at higher pH is probably due to the bacteria, which have a pH optimum at 5 and a markedly decreased activity at pH 7–8.¹ The algae have a pH optimum at 7.² The optimal pH for the system studied here was 6, but pH 4 and 5 were almost as good. In the rest of the optimizing experiments pH 6 was used. It should, however, be borne in mind that the pH optimum for the overall reaction is dependent on the ratio between the participating catalytic moieties.⁵

Influence of carbonate concentration

At low carbonate concentrations the dihydroxyacetone production rate increased linearly with the carbonate

concentration (Figure 3). At higher carbonate concentrations the curve levelled off and it reached a maximum at 30 mM. The rather low production rate at carbonate concentration of 100 mM might be due to secondary effects. At this high concentration of carbonate the buffering capacity of the medium was not sufficient with the result that the pH of the effluent from the column was 6.8. Another effect of the high carbonate concentration was a considerable evolution of carbon dioxide, which probably impaired the flow conditions in the column. Furthermore, it must be realized that a high partial pressure of carbon dioxide may be toxic to bacteria.⁸ In the rest of the optimizing experiments a carbonate concentration of 30 mM was used.

Effect of the light intensity on the overall rate

The optimal light intensity was 20.5 klx (Figure 4). The oxygen production by *Chlorella pyrenoidosa* does not show saturation below 33.5 klx.² The decreased dihydroxyacetone production rate at high light intensity is not yet fully understood.

Effect of variation of the amount of bacteria in the reactor

The dihydroxyacetone production rate of columns containing a constant amount of algae and varying amounts of bacteria was measured. When the amount was small the productivity was directly proportional to the dry weight of bacteria in the reactor (Figure 5). However, the curve levelled off and at bacteria densities exceeding 3.5 mg (dry weight) per ml reactor volume no further increase in dihydroxyacetone production rate occurred. This amount of bacteria is apparently sufficient to utilize the oxygen produced by the algae under the conditions used.

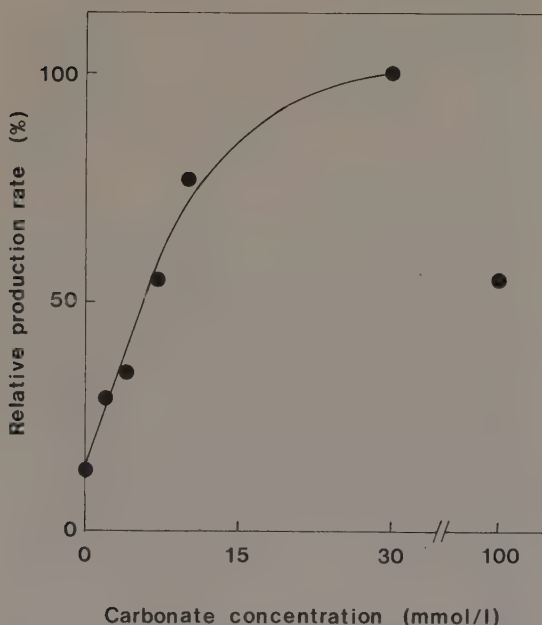


Figure 3 Effect of carbonate concentration on dihydroxyacetone production rate of coimmobilized *Chlorella pyrenoidosa* and *Gluconobacter oxydans*. Maximal productivity was set at 100%

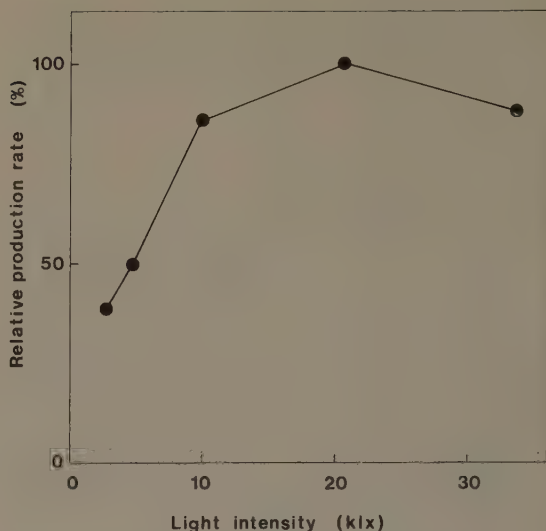


Figure 4 Effect of light intensity on dihydroxyacetone production rate of coimmobilized *Chlorella pyrenoidosa* and *Gluconobacter oxydans*. Maximal productivity was set at 100%

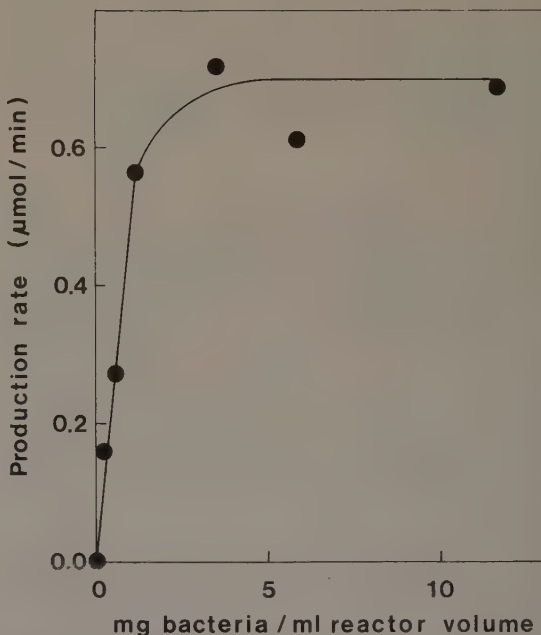


Figure 5 Dihydroxyacetone production rate as a function of the amount of bacteria with a constant amount of algae in a coimmobilized *Chlorella pyrenoidosa*–*Gluconobacter oxydans* preparation

Effect of variation of the amount of algae in the reactor

With a constant amount of bacteria, the dihydroxyacetone production rate increased with the amount of algae up to about 10 mg (dry weight) of algae per ml reactor volume (Figure 6). Further increase of the amount of algae did not increase the production further. This experiment and the preceding one show that in the reactor there are saturation effects with respect to the amount of algae and the amount of bacteria, respectively. Under the conditions

used 10 mg algae ml⁻¹ reactor volume utilizes the light as efficiently as possible in this system, and the oxygen produced can be efficiently utilized by 3.5 mg bacteria ml⁻¹ reactor volume. These figures will change if the experimental conditions (for example light intensity) are altered. Increasing the cell density further does not give any increased productivity in short term experiments, but it might be beneficial in long term experiments if the specific activity of the cells gradually decreases. Anyway, high cell densities do not lower the productivity of the preparations. In long term experiments reactors containing 17 mg algae and 12.5 mg bacteria per ml reactor volume were used.

Storage stability

Under the conditions used, a maleate-containing buffer at pH 6 is a better storage buffer than a succinate-containing buffer at pH 5 (Figure 7). During the first two days of storage in maleate buffer the activity of the preparation increased. No explanation can be offered for this increase. The number of algal cells did not increase during this period.

The storage stability is quite good. After 14 days in the maleate buffer the activity was higher than immediately after immobilization. In the succinate buffer the activity was fairly constant during the period.

Testing long-term dihydroxyacetone production

In preliminary experiments we tested the long-term stability of the coimmobilized system in the medium found to be optimal in the preceding experiments. However, in this medium the operational stability was rather poor. It was found that the succinate based medium used in the experiments on storage stability (supplemented with 30 mM carbonate and 30 mM glycerol) gave a somewhat better stability of the preparation, although the initial activity was lower than that in the optimal medium. Succinate based media at pH 5 was used in the rest of the long-term experiments.

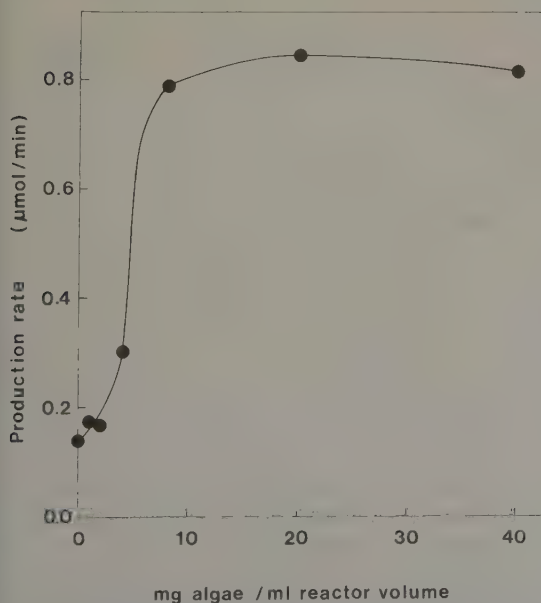


Figure 6 Dihydroxyacetone production rate as a function of the amount of algae with a constant amount of bacteria in a coimmobilized *Chlorella pyrenoidosa*–*Gluconobacter oxydans* preparation

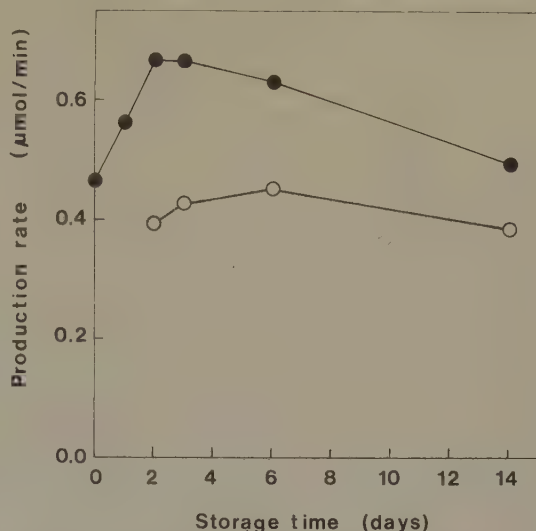


Figure 7 Storage stability of a coimmobilized preparation of *Chlorella pyrenoidosa* and *Gluconobacter oxydans* in a maleate based buffer with pH 6 (●) and in a succinate based buffer with pH 5 (○)

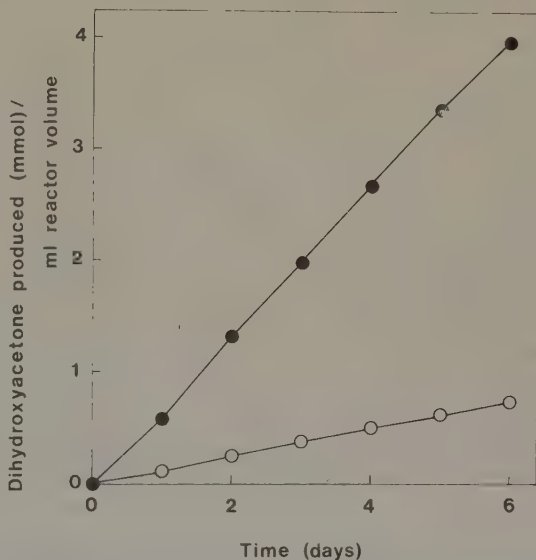


Figure 8 Total amount of dihydroxyacetone produced in a column containing immobilized *Gluconobacter oxydans* cells (○) and a column containing the same amount of bacteria coimmobilized with *Chlorella pyrenoidosa* cells (●)

It was assumed that the decrease in productivity was due to the algae of the preparation. In an effort to reactivate the algae the preparation was treated for short periods (30–40 min) with the culture medium of the algae. With such reactivation the activity of immobilized *Chlorella pyrenoidosa* without bacteria could be maintained for one month.² This treatment increased the dihydroxyacetone production rate. However, the results were even better when a little of the culture medium was added to the production medium. As the culture medium contained phosphate, magnesium and other ions that destabilize the calcium alginate gel, the concentration of calcium chloride in the medium was raised to 20 mM.

With these modifications of the production medium (succinate at pH 5 instead of maleate at pH 6, addition of 5% (v/v) algal culture medium and raising the calcium chloride concentration from 10 to 20 mM), the stability of the preparation was quite good.

During the first 24 h of the experiment, the productivity of the coimmobilized preparation increased, perhaps due to growth of algae on the surface of the gel beads. During the rest of the experiment the dihydroxyacetone production rate of the coimmobilized preparation was fairly constant (Figure 8). Owing to intense growth of algae the column was plugged after operation for 6 days. The activity of the preparation was still quite good. If the superfluous algae had been washed away earlier, the experiment could probably have been continued much longer.

The activity of the column without algae showed a constant activity during the experiment. The accumulated amount of dihydroxyacetone from the column with algae was 5.4 times that from the column without algae.

General discussion

The use of coimmobilized microorganisms is a special variant of mixed cultures. They possess unique metabolic potentials in the sense that a mixture of organisms with complementary metabolic pathways can be used. The mechanisms regulating such mixed cultures have been studied intensively and several general principles have been revealed. These principles deal mainly with growing cultures and thus concern the regulation of the relative numbers of different cells.

Those studies contain much useful information about e.g. ratios between participating cell species in the co-immobilized cell preparation.

An ideal immobilized cell preparation would be one in which the metabolic pathways necessary for the conversion to take place operate at full capacity, while cell growth is limited to an absolute minimum. Such a situation is quite different from that known in mixed culture fermentation technology. It can therefore be foreseen that different criteria will govern the interactions between the participating cell species in the immobilized preparation.

So far, very little has been done in the area of micro-environmental effects on immobilized cell species. With reference to the situation in immobilized enzyme preparations it can be expected that the changed microenvironmental conditions will also influence the metabolic activity of the cell.

To our knowledge this report is the first where coimmobilized cell preparations have successfully been designed to create a favourable microenvironment and thus a good multistep catalyst. Perlman and coworkers⁹ tried this approach in their effort to produce 2-keto-L-gulonate, but found separate immobilization to be superior to coimmobilization.

When immobilizing a mixed culture, very high cell densities may be maintained, in some cases higher than what is practically feasible in free solution. Furthermore, the physiological conditions of immobilized cells might have changed from those in free solution. Some indications point towards a lower growth rate and a higher productivity for which reason one can assume that the rules for regulation between the different species in a coimmobilized preparation differ from those in free solution.¹⁰

As mentioned earlier, the microenvironment created in the solid phase differs from that in the bulk phase. These

differences may be ascribed to the matrix itself, but also to enrichment of metabolites from the entrapped cells. The importance of such an environment for immobilized enzymes has been studied and explained in some detail but not for immobilized cells.

As stated earlier² immobilization in alginate is not the best choice for this application.

Use of photosynthetic reactions for oxygen generation places very specific demands on the design of the reactor.

Symbionts of the type discussed in this paper are special cases of mixed cultures, in that the two participating cell species are often coordinated in more than one way. In the natural symbionts, e.g. lichens an integrated metabolic pattern is known to exist with specific functions for each of the participating cell species. Such a degree of integration is not expected in an artificial symbiont, but interactions involving more than one or a few common intermediates may be expected.

The interaction may be positive as well as negative. *Chlorella* is known to generate toxic peroxides of lipids^{11,12} but no evidence of any effect of such substances was seen in the present study. *Gluconobacter* must in this context be regarded as a model system to test the effect of coimmobilized green algae. In future applications it may be of interest to use oxygen consumers which produce carbon dioxide and thereby create possibilities for a favourable microenvironment for both metabolic entities.

The use of algae to generate oxygen within immobilized cell systems offers certain advantages. Thanks to the *in situ* generation, it is possible to maintain a uniform oxygen tension also in packed bed reactors. This was earlier found to be a problem when hydrogen peroxide was used as an oxygen source.¹ Furthermore, since oxygen in too high a concentration may be toxic it should be stressed that the use of photolysis provides a possibility to control the oxygen tension by regulating the light intensity.

Acknowledgements

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References

- Holst, O., Enfors, S.-O. and Mattiasson, B. *Eur. J. Appl. Microbiol. Biotechnol.* 1982, **14**, 64–68
- Adlercreutz, P. and Mattiasson, B. *Enzyme Microb. Technol.* 1982, **4**, 332–336
- Mattiasson, B. *Doctoral Thesis* University of Lund (1974)
- Mattiasson, B. 1977. in *Biomedical Applications of Immobilized Enzymes and Proteins* (T. M. S. Chang, ed.) Plenum Press, New York, vol. 2, pp. 253–269
- Gestrelus, S., Mattiasson, B. and Mosbach, K. *Biochim. Biophys. Acta* 1972, **276**, 339–343
- Starr, R. C. *Am. J. Bot.* 1964, **51** (9), 1013–1044
- Müller, G. L., Blum, R., Glennon, W. E. and Burton, A. L. *Anal. Biochem.* 1960, **2**, 127–132
- Enfors, S.-O. and Molin, G. *J. Appl. Bacteriol.* 1978, **45**, 279–285
- Martin, C. K. A. and Perlman, D. *Eur. J. Appl. Microbiol. Biotechnol.* 1976, **3**, 91–95
- Navarro, J. M. and Durand, G. *Eur. J. Appl. Microbiol. Biotechnol.* 1977, **4**, 243–254
- Robertson, P., Daniels, T. C., Eiler, J. J., Gunnison, J. B., Kumler, W. D., Oneto, J. F., Strait, L. A., Spoer, H. A., Hardin, G. J., Milner, H. W., Smith, J. H. C. and Strain, H. H. *Science* 1944, **99**, 351–352
- Spoer, H. A., Smith, J. H. C., Strain, H. H., Milner, H. W. and Hardin, G. J. *Carnegie Inst. Wash. Pub. No 586*, 1949

Oxygen Supply to Immobilized Cells

3. Oxygen Supply by Hemoglobin or Emulsions of Perfluorochemicals*

Patrick Adlercreutz and Bo Mattiasson

Department of Pure and Applied Biochemistry, Chemical Center, University of Lund, P.O. Box 740, S-22007 Lund, Sweden

Summary. Oxygen supply is a critical point in technical processes when aerobic cells are used in immobilized preparations. This report concerns the use of hemoglobin or emulsions of perfluorochemicals (completely fluorinated organic compounds) to carry oxygen to immobilized cells. Both methods work well and do not seem to harm the cells. The perfluorochemical method of improving oxygen supply showed a higher operational stability than the hemoglobin method.

Introduction

Oxygen is required by many organisms. When microorganisms are used in technical processes, the supply of oxygen is often a critical point (Enfors and Mattiasson in press). In conventional fermentation technology oxygen is often supplied by bybbling air, or occasionally pure oxygen, through the fermentor. Vigorous stirring of the medium is necessary to get good oxygen transfer. When immobilized biocatalysts are used in packed bed reactors these methods cannot be used, so other methods must be developed (Adlercreutz et al. 1982; Holst et al. 1982; Tramper and Van den Tweel 1981).

Some increase in oxygen supply can be obtained by increasing the flow rate of the medium. However, this is often not sufficient to get an acceptable reaction rate, since the solubility of oxygen in water solutions is very low. Furthermore, this method would give a very dilute solution of the product, which is undesirable in downstream processing. Problems with oxygen supply are especially pronounced when high biocatalyst densities are used, such as in immobilized preparations.

Different methods have been used to increase the oxygen supply to immobilized biocatalysts. Among these, there are some methods where oxygen is generated in situ. This can be done, for example, by adding hydrogen peroxide to the medium and using an organism with a high natural catalase activity. This method has been used to supply oxygen to the bacteria *Gluconobacter oxydans* (Holst et al. 1982). Another way to generate oxygen in situ is to co-immobilize oxygen-consuming organisms with oxygen-producing organisms, for example algae. This has been studied in a *Chlorella pyrenoidosa*-*Gluconobacter oxydans* preparation (Adlercreutz et al. 1982).

Still another approach is to modify the medium in such a way that it dissolves more oxygen. The water solutions normally used are replaced by organic solvents, with the intention of increasing the solubility of both organic substrates (steroids) and oxygen (Buckland et al. 1975).

This report concerns addition of certain substances to an aqueous medium in order to increase its capacity to transfer oxygen to the biocatalyst in the reactor. In nature several substances capable of transporting oxygen have been found. The most well-known is hemoglobin. One part of this report deals with the use of hemoglobin to supply immobilized cells with oxygen. In this case, the role of hemoglobin is not very different from its normal role in animals.

The other part of this report concerns the use of perfluorochemical emulsions to supply oxygen to immobilized cells. Perfluorochemicals are organic compounds in which all hydrogen atoms have been replaced by fluorine atoms (Fig. 1). They are very nonpolar, heat-stable and very inert chemically. Many gases, for example oxygen and carbon dioxide, have a high solubility in perfluorochemicals. These properties make perfluorochemicals suitable as blood substitutes. In this application, pure perfluorochemicals cannot be used, but emulsions of

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Offprint requests to: P. Adlercreutz

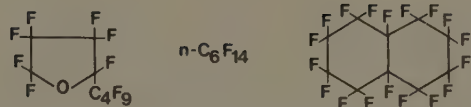


Fig. 1. Some examples of perfluorochemicals

these have successfully been used in "blood-transfusions" to several different animals (Riess and Le Blanc 1978) and, recently, to humans (Maugh 1979; Lundsgaard-Hansen 1980). To our knowledge, this report is the first one where perfluorochemicals have been used to increase the oxygen supply to microorganisms.

As an example of an oxygen consuming organism the acetic acid bacteria *Gluconobacter oxydans* was used. In the presence of oxygen these bacteria are able to convert glycerol to dihydroxyacetone. The amount of dihydroxyacetone produced was used as an indicator of the oxygen supply in the preparation.

Materials and Methods

Materials. Sodium alginate was purchased from KEBO AB, Malmö, Sweden and bacto-agar from Difco, Detroit, MI USA. Hemoglobin was prepared from pig erythrocytes (see below). Pluronic F-68 was purchased from Montoil AB, Stockholm, Sweden, and the perfluorochemical FC-72 from 3M, St. Paul, MN, USA. FC-72 is a mixture of different perfluorinated compounds. The main constituent is C_6F_{14} , perfluorinated hexane. All other chemicals were of analytical grade.

Organism. The bacteria *Gluconobacter oxydans* (ATCC 621) was cultivated as previously described (Holst et al. 1982).

Preparation of Hemoglobin. Pig erythrocytes were washed 3 times with an isotonic NaCl-solution and then lysed in distilled water. The erythrocyte membranes were removed by centrifugation and the hemoglobin-containing supernatant was then concentrated in a hollow-fiber unit.

Immobilization. Cells were immobilized in calcium alginate gel as described earlier (Adlercreutz and Mattiasson 1982a). The gel beads were stored at 4 °C in one of the following three 0.1 M buffer solutions: pH 5: succinate-, pH 6: maleate-, pH 7: Tris-HCl-, all containing 10 mM $CaCl_2$.

Preparation of Emulsions of Perfluorochemicals. The buffer solution (water phase), the perfluorochemical FC-72 (organic phase) and Pluronic F-68 (emulgator) were mixed in appropriate proportions. The amount of Pluronic F-68 was always 0.21 g per ml FC-72. The mixture (50 ml each time) was poured into a 400 ml beaker which was cooled with crushed ice. The mixture was then sonicated using the probe of a A350G sonicator from Ultrasonics Ltd, England (5 min, 350 W). A white emulsion formed.

Experiments with Immobilized Bacterial Cells. Oxygen transfer to whole cells was tried with the bacteria *Gluconobacter oxydans*. These bacteria oxidize glycerol to dihydroxyacetone with

oxygen as acceptor of the eliminated hydrogen. The bacteria were immobilized in calcium alginate gel which was then packed in glass columns (I.D. 5.5 mm). A buffer solution containing calcium ions to stabilize the alginate beads and glycerol as a substrate for the bacteria was pumped through the columns. The concentration of dihydroxyacetone in the effluents of the columns was measured (see below). The effect of the addition of hemoglobin or perfluorochemical emulsions to the medium was investigated.

In some experiments the medium was recirculated. The medium was kept in a beaker, stirred magnetically, and continuously pumped through the column reactor back to the beaker. In these experiments samples were taken regularly from the beaker.

In the experiments with perfluorochemicals the FC-72 emulsion was kept in a bottle and was stirred magnetically. The bottle was connected to open atmosphere via a narrow tube to equilibrate the pressure when emulsion was pumped from the bottle to the reactor. In some of the experiments the emulsions were saturated with pure oxygen. The stream of oxygen gas was led through a bottle containing a mixture of water and FC-72 to presaturate the gas and then to a flask where the emulsion to be used was placed. In this way compensation for losses by the off-gas was achieved.

Quantification of Hemoglobin. Total concentrations of hemoglobin were determined as hemiglobincyanide (Anonymous 1965). The concentrations of oxyhemoglobin and methemoglobin were determined from absorbance measurements at 576 and 630 nm using the equations of Benesch et al (1973). All preparations used contained mainly oxyhemoglobin but also some methemoglobin. The methemoglobin did not contribute to the oxygen-transporting capacity of the medium. In this text the concentration of oxyhemoglobin is given.

Identification and Quantification of Dihydroxyacetone. Dihydroxyacetone was identified on high performance thin layer chromatography (HPTLC) plates as previously described (Holst et al. 1982). Quantification of dihydroxyacetone was done with the DNS-method of Miller et al. (1960). The samples had to be specially treated before analysis.

When hemoglobin-containing solutions were analyzed, 1 ml of a 33% solution of $Pb(NO_3)_2$ was added to 1 ml of the sample solution (containing not more than 150 mg hemoglobin) in a test tube. The test tubes were shaken vigorously so that the precipitation of hemoglobin was complete. After centrifugation, 1 ml of the clear solution was added to 0.4 ml saturated NaCl-solution. The lead chloride precipitate formed was removed by centrifugation and 1 ml of the solution was used for DNS-analysis. Known amounts of dihydroxyacetone were treated like the samples to get a standard curve. This curve was almost identical with that obtained with the standard procedure (when correction was made for the dilution caused by addition of extra reagents).

When the concentration of dihydroxyacetone in perfluorochemical emulsions was determined, the emulsion was centrifuged so that the heavy perfluorochemical phase accumulated in the bottom of the tube and the clear water solution could be used for analysis. The organic phase did not contain any measurable amounts of dihydroxyacetone. The concentration of dihydroxyacetone in the water phase was used to calculate the average concentration in the emulsion.

Test of Cell Viability. A viability test was performed to evaluate potential negative effects of perfluorochemicals on the bacteria. The bacteria were immobilized in calcium alginate gel as in the previous experiments. One portion of the gel beads was put in

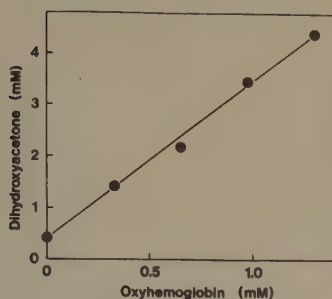


Fig. 2. Effect of medium oxyhemoglobin concentration on the production of dihydroxyacetone by immobilized *Gluconobacter oxydans*. The reactor (vol 2 ml) contained 40 mg immobilized bacteria. A medium containing 100 mM glycerol, 100 mM maleate, 10 mM CaCl_2 and varying amounts of oxyhemoglobin (pH 6.0) was pumped through the reactor at a rate of 0.25 ml/min. The samples were taken between 45 and 60 min after the start of each experiment

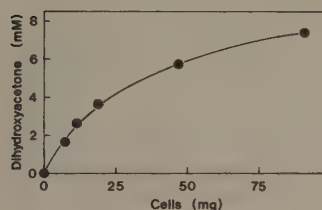


Fig. 3. Dihydroxyacetone production as a function of the amount of immobilized *Gluconobacter oxydans* in the reactor. Oxygen was supplied by oxyhemoglobin. A calcium alginate gel containing 42 mg (dry weight) bacteria per g wet gel was used. Different amounts of the gel was packed in glass columns of appropriate length (I.D. 5.5 mm). A medium containing 100 mM glycerol, 50 mM maleate, 10 mM CaCl_2 and 1.3 mM oxyhemoglobin (pH 6.1) was pumped through the columns at a rate of 0.10 ml/min. Samples were taken between 45 and 60 min after the start of each experiment

0.1 M succinate buffer (pH 5.0) containing 10 mM CaCl_2 . Another portion of the gel beads was put in a 37.5% (v/v) emulsion of FC-72 in the same buffer. The two samples were put into two separate test tubes with screw caps and were incubated on a rocking table for 12 days at room temperature.

For each determination of cell viability 20 gel beads from each tube were dissolved in 0.1 M citrate-phosphate buffer (pH 6.0). The cell suspensions were diluted and spread on agar plates. The agar contained the culture medium of the bacteria (Holst et al. 1982) and 1.5% agar. Colonies were counted after 48h incubation at 28 °C.

Results and Discussion

Oxygen Supply by Hemoglobin to Immobilized Cells

Hemoglobin was used to supply immobilized whole cells, *Gluconobacter oxydans*, with oxygen. The cells were

apparently able to use the extra oxygen supplied by hemoglobin to increase the rate of the oxidation reaction: glycerol $\rightarrow$ dihydroxyacetone. The dihydroxyacetone production varied with the hemoglobin concentration (Fig. 2).

The amount of immobilized bacteria in the reactor was varied. The production of dihydroxyacetone increased with the amount of cells (Fig. 3). No plateau-value was obtained in the range tested (≤ 91 mg dry weight of bacteria). Diffusion restrictions within the immobilized preparation and the high affinity of hemoglobin for oxygen apparently make it necessary to use a large amount of cells if the oxygen supplied is to be effectively utilized.

If hemoglobin is to be used as an oxygen carrier in biotechnical processes it is necessary that the protein can be used several times. It is known that hemoglobin is slowly oxidized by oxygen to methemoglobin. In the red blood cells methemoglobin is continuously reduced to hemoglobin by enzymatic processes.

Recirculation of hemoglobin-containing solutions (containing a large amount of substrate) through a reactor with immobilized bacteria was tried at different pH-values. The bacterial oxidation of glycerol to dihydroxyacetone by *Gluconobacter oxydans* has a pH-optimum at 5 (Holst et al. 1982). However, at pH 5 the oxidation of hemoglobin to methemoglobin is rather rapid so that the production rate decreased after a few hours (results not shown). It is known that hemoglobin is more stable towards oxidation at higher pH-values (Mansouri and Winterhalter 1973). Recirculation experiments were therefore done at pH 6 and 7. The fastest production of dihydroxyacetone was obtained at pH 6 (Fig. 4). Some decrease in production rate, due to oxidation of hemoglobin, was observed. At the end of the experiment the concentration of oxyhemoglobin in the medium was 0.38 mM compared to 0.85 mM at the start. The rest had been oxidized to methemoglobin.

At pH 7 the decrease in productivity was more pronounced than at pH 6. This was probably due to decreased efficiency in the bacterial production of dihydroxyacetone. The oxidation of hemoglobin was rather slow. The initial concentration of 0.85 mM oxyhemoglobin had been reduced to 0.63 mM by the end of the experiment. These results show that the use of hemoglobin as an oxygen carrier is more favourable at pH-values above 6. The process used here is thus not an ideal example.

The oxidation of hemoglobin can be reversed. There are several reports concerning this reaction. Different reducing agents such as ascorbic acid (Tomoda et al. 1976, 1978) and iron (III) EDTA-complex (Mauk and Gray 1979) have been used. Preliminary experiments using these reagents showed that methemoglobin was reduced to hemoglobin although the reaction could not be driven

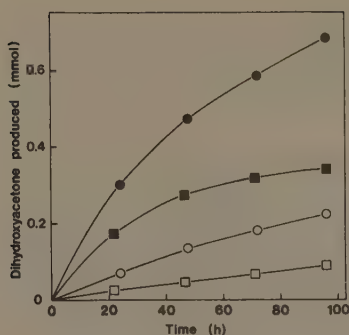


Fig. 4. Continuous production of dihydroxyacetone by immobilized *Gluconobacter oxydans* in hemoglobin-containing solutions. 15 ml of solutions containing 0.85 mM oxyhemoglobin, 10 mM CaCl_2 and 100 mM maleate (pH 6.0; ●) or 100 mM Tris-HCl (pH 7.0; ■) were poured into beakers and then recirculated at a flow rate of 0.10 ml/min through reactors (vol 2 ml) containing 27 mg immobilized bacteria. The solutions in the beakers were stirred magnetically. As blanks, two reactors were treated as above except that their media contained no hemoglobin (pH 6.0: ○ and pH 7.0: □)

to completeness. Further work is required to make these reactions practically useful.

Oxygen Supply by Perfluorochemical Emulsions to Immobilized Cells

The effect of addition of perfluorochemicals to the medium when immobilized *Gluconobacter oxydans* were used to oxidize glycerol to dihydroxyacetone was very obvious. The productivity increased with the amount of FC-72 in the medium (Fig. 5). When the medium was saturated with oxygen the productivity was 4–5 times that obtained with air-saturated media.

To check the degree of oxygen saturation, the oxygen pressure in the emulsions was measured with a polarographic oxygen electrode (Radiometer). Probably because of the relatively high vapour pressure of FC-72 at room temperature (FC-72 has a boiling point of 56 °C (3M 1979)) oxygen pressures above 76 kPa could not be obtained in the emulsions, whereas buffer solutions could be saturated to give oxygen pressures of 100 kPa. Another possibility is that the apparently low oxygen pressure measured in the emulsions was due to their high viscosity. It is known that the viscosity of a solution influences the measurement read from a polarographic oxygen electrode to some extent. Prolonged "saturation times" before the use of emulsions in the reactor should be avoided because of the potential experimental error caused by evaporation and subsequent loss of FC-72. To diminish this risk

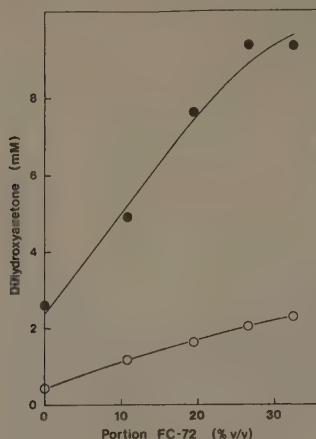


Fig. 5. Effect of the amount of perfluorochemicals (FC-72) in the medium on the production of dihydroxyacetone by immobilized *Gluconobacter oxydans*. The reactors (vol 7 ml) contained 230 mg immobilized bacteria. FC-72 was emulgated by sonication in 0.1 M succinate buffer (pH 5.0) containing 0.25 M glycerol and 10 mM CaCl_2 using Pluronic F-68 as an emulgator. The emulsions were saturated with air (○) or oxygen (●). The emulsions were pumped through the reactors at a flow rate of 0.20 ml/min. Samples were taken between 80 and 110 min after the start of each experiment. The concentration of dihydroxyacetone was measured in the water phase and recalculated to give the concentration of dihydroxyacetone in the emulsion

the oxygen gas was forced through a mixture of water and FC-72 before coming into contact with the emulsions.

Furthermore, analysis with polarographic oxygen electrodes to determine the total transport capacity of oxygen seems insufficient. Instead, a method based on oxygen consumption by an enzyme reaction monitored in a simple flow-calorimeter was used (Adlercreutz and Mattiasson 1982b).

A high portion of perfluorochemicals in the emulsion is beneficial in the sense that it gives a high production rate, but emulsions containing more than 35% (v/v) FC-72 were difficult to handle because of their high viscosity.

When the amount of immobilized *Gluconobacter oxydans* in the reactor was varied, the productivity increased with the amount of bacteria up to about 50 mg dry weight of bacteria (Fig. 6). The curve then levelled off and further increases of the amount of bacteria did not effect the productivity of the reactor, probably because 50 mg of bacteria could utilize all the oxygen supplied in the medium. This value is, of course, dependent on the composition and the flow rate of the medium.

To evaluate the operational stability of immobilized *Gluconobacter oxydans* in an emulsion of FC-72 a seven day experiment was performed. Dihydroxyacetone was produced at an almost constant rate throughout the ex-

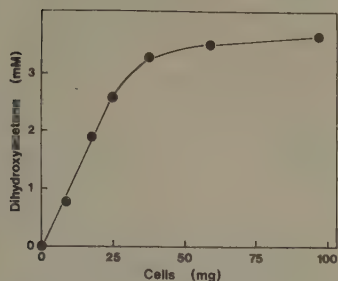


Fig. 6. Dihydroxyacetone production as a function of the amount of *Gluconobacter oxydans* in the reactor. Oxygen was supplied by air-saturated emulsions of FC-72. The bacteria were immobilized in calcium alginate gel (32 mg dry weight of bacteria per g wet gel), and packed in columns (I.D. 5.5 mm) of appropriate length. A 32.4% (v/v) emulsion of FC-72 in 0.1 M succinate buffer (pH 5.0) containing 0.25 M glycerol and 10 mM CaCl_2 was pumped through the columns at a rate of 0.20 ml/min. Samples were taken from the effluents of the columns between 45 and 60 min after the start of each experiment

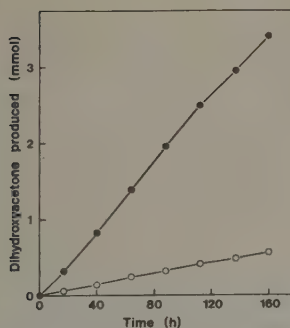
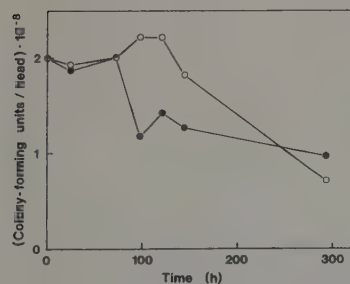


Fig. 7. Operational stability of immobilized *Gluconobacter oxydans* in a perfluorochemical emulsion. A 32.4% (v/v) emulsion of FC-72 in 0.1 M succinate buffer containing 0.25 M glycerol and 10 mM CaCl_2 (pH 5.0) was continuously pumped through a reactor (vol 3.5 ml) containing 105 mg immobilized bacteria. The flow rate was 0.10 ml/min and the emulsion was saturated with air. Samples were taken regularly from the effluent of the column and the accumulated amount of dihydroxyacetone was calculated (●). An identical column was treated in the same way except that FC-72 and the emulgator Pluronic F-68 were omitted (○)



periment (Fig. 7). The accumulated amount of dihydroxyacetone was 6 times that of an identical column treated with a medium that did not contain FC-72. This shows that FC-72 is very useful as an oxygen carrier in this system. Apparently the cells retained their metabolic activity throughout the experiment. In order to check if the bacteria also retained their viability a 12 day experiment was performed. One portion of alginate immobilized *Gluconobacter oxydans* was incubated in succinate buffer and another portion in an emulsion of FC-72. The number of viable cells decreased slowly both in the buffer solution and in the perfluorochemical emulsion (Fig. 8). Apparently the emulsion of FC-72 was very biocompatible. After 12 days there was no significant difference between the number of viable cells in beads incubated in the emulsion compared to beads incubated in buffer.

It has earlier been shown that perfluorochemicals are very biocompatible in animals (Riess and Le Blanc 1978; Maugh 1979; Lundsgaard-Hansen 1980) and it is therefore not surprising that they are also biocompatible in microbial systems.

General Discussion

All the methods for supplying oxygen to immobilized biocatalysts have advantages and disadvantages. It is not yet possible to say which method is best. An important characteristic is the maximal amount of oxygen which can be transferred to the biocatalyst by a certain method. In this respect the two methods described in this report are approximately equal. The amount of oxygen-carrying substance is, of course, of great importance. When solutions of oxygen-carrying substances are used, the solubility of the oxygen-carrier in the buffer used sets a limit for the oxygen transporting capacity of the medium. Hemoglobin is very soluble, but working with solutions much more concentrated than the 85 mg/ml used in this study does not seem realistic.

When emulsions of perfluorochemicals are considered other characteristics, such as viscosity, set the limit for the amount of oxygen carrying substance in the medium. With the techniques used in this study emulsions containing more than 35% (v/v) FC-72 were difficult to handle.

Among other methods, oxygen supply from degraded hydrogen peroxide gives a very high initial reaction rate (Holst et al. 1982). However, this method cannot yet be successfully applied in long-term runs because the activity of the preparation decreases rapidly. The life span of

◀ Fig. 8. Number of viable *Gluconobacter oxydans* cells as a function of incubation time in 0.1 M succinate buffer (pH 5.0) containing 10 mM CaCl_2 (○) and in a 37.5% (v/v) emulsion of FC-72 in the same buffer (●). The cells were immobilized in calcium alginate gel

the immobilized preparation is of great importance. A good operational stability has been reported for a co-immobilized algae-bacteria preparation where oxygen was supplied by the algae (Adlercreutz et al. 1982).

In general, organic solvents can be assumed to damage the membranes of the immobilized cells. The use of emulsions instead of pure organic solvents reduces this risk.

Perfluorochemicals appear to be more biocompatible than most other organic solvents. In the present study the operational stability of immobilized *Gluconobacter oxydans* in an emulsion of FC-72 was quite good. Hemoglobin probably does not harm the cells, but there is another problem. To make a process economically realistic it would probably be necessary to re-use the oxygen-transporting substance several times. Hemoglobin is oxidized to methemoglobin at a slow but significant rate. As a result of this oxidation, the oxygen transporting capacity of the medium gradually decreases. But, as mentioned above, there are possibilities to reverse this reaction. Perfluorochemicals are chemically very inert and can probably be re-used many times. The recovery procedure for the oxygen transporting substance is of some importance. Perfluorochemicals can, because of their high density, easily be recovered (e.g., by centrifugation). In the case of hemoglobin, ultra-filtration or similar methods must be used.

An important feature of an oxygen-supplying method is the ease with which oxygen is released in the reactor. Oxygen is dissolved in perfluorochemical emulsions in direct proportion to the partial pressure of oxygen in the gas phase used to saturate the emulsion. When the biocatalyst in the reactor uses oxygen in the water phase of the emulsion oxygen is easily released from the perfluorochemical phase. In contrast to perfluorochemicals, hemoglobin has a high affinity for oxygen and therefore it is necessary for the medium to have a long residence time in the reactor if the oxygen supplied by the hemoglobin is to be effectively utilized. Another alternative is to use a larger amount of biocatalyst in the reactor.

Perfluorochemicals are good solvents not only for oxygen but also for many other gases. One important example is carbon dioxide. This gas is often produced in aerobic bioprocesses and is inhibitory to many organisms. In addition to carrying reaction-promoting gases to the reactor, it is probable that perfluorochemicals can be used to carry inhibitory gases from the reactor as well. Furthermore, due to their high biocompatibility, perfluorochemicals are an ideal choice for systems where living cells must be exposed to organic solvents.

In conclusion, both hemoglobin and perfluorochemical emulsions can be successfully used to supply oxygen to immobilized biocatalysts. Neither hemoglobin nor perfluorochemical emulsions seem to harm the cells.

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References

- Adlercreutz P, Mattiasson B (1982a) Oxygen supply to immobilized cells: 1 Oxygen production by immobilized *Chlorella pyrenoidosa*. *Enzyme Microbial Technol* 4:332–336
- Adlercreutz P, Mattiasson B (1982b) Oxygen supply to immobilized biocatalysts. A model study. *Acta Chem Scand B* 36:651–653
- Adlercreutz P, Holst O, Mattiasson B (1982) Oxygen supply to immobilized cells: 2. Studies on a co-immobilized algae-bacteria preparation with in situ oxygen generation. *Enzyme Microbial Technol* 4:395–400
- Anonymous (1965) Recommendations and requirements for haemoglobinometry in human blood. *Nature* 206:491–492
- Benesch RE, Benesch R, Yung S (1973) Equations for the spectrophotometric analysis of hemoglobin mixtures. *Anal Biochem* 55:245–248
- Buckland BC, Dunnill P, Lilly MD (1975) The enzymatic transformation of water-insoluble reactants in nonaqueous solvents. Conversion of cholesterol to cholest-4-ene-3-one by a *Nocardia* sp. *Biotechnol Bioeng* 17:815–826
- Enfors SO, Mattiasson B (in press) Oxygenation of processes involving immobilized cells. In: Mattiasson B (ed) *Immobilized cells and organelles*. CRC-Press, London
- Holst O, Enfors S-O, Mattiasson B (1982) Oxygenation of immobilized cells using hydrogen-peroxide; A model study of *Gluconobacter oxydans* converting glycerol to dihydroxyacetone. *Eur J Appl Microbiol Biotechnol* 14:64–68
- Lundsgaard-Hansen P (1980) Synthetisches Blut. *Dtsch Med Wochenschr* 105:1197–1198
- Mansouri A, Winterhalter KH (1973) Nonequivalence of chains in hemoglobin oxidation. *Biochemistry* 12:4946–4949
- Maugh TH (1979) Blood substitute passes its first test. *Science* 206:205
- Mauk AG, Gray HB (1979) Analysis of the kinetics of electron transfer reactions of hemoglobin and myoglobin with inorganic complexes. *Biochem Biophys Res Commun* 86:206–210
- Miller GL, Blum R, Glennon WE, Burton AL (1960) Measurement of carboxymethylcellulase activity. *Anal Biochem* 2:127–132
- Riess JG, Le Blanc M (1978) Perfluor-Verbindungen als Blutersatzmittel. *Angew Chem* 90:654–668
- Tomoda A, Matsukawa S, Takeshita M, Yoneyama Y (1976) Effect of organic phosphates on methemoglobin reduction by ascorbic acid. *J Biol Chem* 251:7494–7498
- Tomoda A, Takeshita M, Yoneyama Y (1978) Characterization of intermediate hemoglobin produced during methemoglobin reduction by ascorbic acid. *J Biol Chem* 253:7415–7419
- Tramper J, Van den Tweel WJJ (1981) Continuous production of gluconic acid by an immobilized *Gluconobacter oxydans* system. *Sec Eur Congr Biotechnol Abstracts*, p 161
- 3M (1979) Fluorinert electronic liquids

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Oxygen supply to immobilized cells

4. Use of p-benzoquinone as an oxygen substitute

Patrick Adlercreutz and Bo Mattiasson

Department of Pure and Applied Biochemistry, Chemical Centre, University of Lund,
P.O. Box 740, S-220 07 Lund, Sweden

Summary. Oxygen supply is a critical point in technical processes when aerobic cells are used in immobilized preparations. In this study p-benzoquinone is used as a substitute for oxygen in the oxidation of glycerol to dihydroxyacetone by immobilized *Gluconobacter oxydans* cells. The reaction rate was much higher when p-benzoquinone was used compared to when oxygen was used. In an experiment with free cells p-benzoquinone gave a rate more than four times that of oxygen, and with immobilized cells the difference was even greater. p-benzoquinone is more effective than oxygen because it gives a higher maximal reaction rate (the reason for this fact is discussed) and because it is more soluble in water than oxygen. The operational stability of the process is comparatively good. In one experiment the productivity decreased from 60 to 10 mmol/h · g over an 8-day period when p-benzoquinone was used. When oxygen was used in a similar experiment the productivity decreased from 14 to 6 mmol/h · g. The byproduct formed from p-benzoquinone, hydroquinone, can be oxidized to p-benzoquinone which can be re-used. Seven successive regenerations of p-benzoquinone were performed without any loss of efficiency.

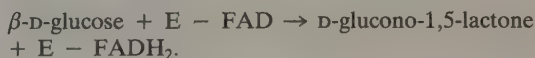
Introduction

Oxygen is required by many microorganisms. When microorganisms are used in technical processes the supply of oxygen is often a critical point (Enfors and Mattiasson 1983). One reason is the low solubility of oxygen in water solutions. If the cells are immobilized before bioconversion takes place the problems concerning oxygen supply get even more severe. This is

due to the extra resistance to oxygen transport: oxygen must diffuse through the matrix to reach the cells.

Several methods have been tried to increase the oxygen supply to immobilized biocatalysts. Oxygen has been added as hydrogen peroxide which is decomposed by catalase in the reactor (Holst et al. 1982). Another way to generate oxygen in the reactor is to co-immobilize the oxygen consuming organism with oxygen producing organisms, for example algae (Adlercreutz et al. 1982). The water solutions normally used can sometimes be replaced by organic solvents which dissolve more oxygen and also more organic substrates (steroids) (Buckland et al. 1975). Other ways to increase the oxygen content of the medium are to add hemoglobin or emulsions of perfluorochemicals (Adlercreutz and Mattiasson 1982).

In this report an entirely different approach is presented. It has been known for a long time that many enzymes that normally use cytochromes or oxygen as electron acceptors can also use artificial electron acceptors such as 2,6-dichlorophenol-indophenol, ferricyanide and other compounds. One example is D-glucose-oxidase (E.C. 1.1.3.4). This enzyme is a flavoprotein containing FAD as a cofactor. In the first step of the enzymatic reaction β -D-glucose is oxidized by the oxidized form of the enzyme:



The reduced form of the enzyme is re-oxidized by oxygen:



In this reaction oxygen can be replaced by artificial electron acceptors such as 2,6-dichlorophenol-indo-

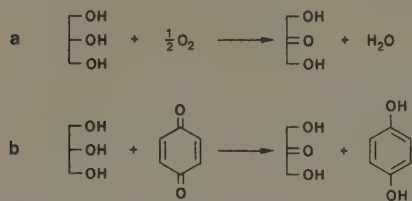


Fig. 1a, b. Formulas for the production of dihydroxyacetone from glycerol by *Gluconobacter oxydans* cells. In the normal reaction (a) oxygen is used as electron acceptor, but artificial electron acceptors such as p-benzoquinone (b) can replace oxygen. p-Benzoquinone is reduced to hydroquinone

phenol (Barman 1969). It has recently been shown that also p-benzoquinone works as an electron acceptor in the reaction above (Alberti and Klivanov 1982).

In this study p-benzoquinone was tried as an oxygen substitute in a reaction catalyzed by whole cells. The bacteria *Gluconobacter oxydans* are able to oxidize glycerol to dihydroxyacetone. For this reaction the cells normally use oxygen (Fig. 1a). However, this study shows that p-benzoquinone is an efficient oxygen substitute in this reaction (Fig. 1b).

Materials and methods

Materials. Sodium alginate (Type IV lot no. 111F-0049) and horse radish peroxidase (Type I) were purchased from Sigma, p-benzoquinone (zur Synthese) and hydrogen peroxide (Perhydrol zur Analyse) from Merck and Sepharose 4B from Pharmacia.

Organism. *Gluconobacter oxydans* (ATCC 621) was grown as previously described (Holst et al. 1982).

Immobilization. An alginate solution containing 2.5% (w/w) sodium alginate in 0.9% NaCl was prepared. The bacterial cells were suspended in 0.9% NaCl solution. A mixture was made to get the desired cell density and a final alginate concentration of 1.5% (w/w). The mixture was pumped through a hypodermic needle and dropped into a 0.1 M CaCl_2 -solution. The surface of this solution was about 10 cm below the needle. Beads formed immediately. The beads were cured in the CaCl_2 -solution for 2 h. Then the beads were filtered off and stored in 0.1 M succinate buffer (pH 5.0) containing 10 mM CaCl_2 at 4°C until use. The diameter of the beads was 2.2 mm. The cell density in the beads is expressed as grams of cells (dry weight) per liter gel (volume of the beads).

Bioconversion experiments. All experiments were carried out in 0.1 M succinate buffer (pH 5.0) containing 10 mM CaCl_2 if not otherwise stated. p-Benzoquinone and glycerol were dissolved in the buffer to get a substrate solution. This solution was filtered through a filter paper to remove insoluble impurities in the p-benzoquinone. Most experiments were carried out in test tubes (vol 17 ml) covered with a rubber membrane and a screw-cap. The substrate solution and the cells (free cells suspended in 0.9% NaCl solution or immobilized cells) were added to the test tubes. The total volume was 15 ml and all substrate concentrations were based

on this volume (the concentrations in the substrate solutions were therefore slightly higher than the concentrations in the experiments). The air in the test tubes was replaced by nitrogen but nitrogen was not bubbled through the solution. The tubes were incubated on a rocking table at 22°C. Samples were taken from the tubes with hypodermic syringes through the membranes. One syringe was used to take the sample (usually 0.4 ml) and another to add an equal volume of nitrogen gas to maintain atmospheric pressure in the tubes.

Analysis of dihydroxyacetone. The samples from the bioconversion experiments (0.4 ml) were immediately extracted with 5 ml diethylether. 200 µl of the water phase was added to 1,800 µl acetonitrile. This procedure took about 1 min. The samples could be stored at this stage at 4°C overnight. Before HPLC-analysis the samples were centrifuged to avoid injection of solid particles into the column. The HPLC-analyses were performed with a 4.5 × 250 mm column packed with Spherisorb W (5 µm). Acetonitrile-water (90 : 10) was used as mobile phase and the flow rate was 0.75 ml/min. A 20 µl sample loop was used. The compounds were detected with a UV-detector at 275 nm. The retention time for dihydroxyacetone was about 4.7 min.

Analysis of hydroquinone and p-benzoquinone. The samples from the bioconversion experiments were mixed with an equal volume of methanol. When analyzing samples from experiments with free cells the samples were injected immediately on the HPLC as the reaction did not stop completely after the addition of methanol. The HPLC-analyses were performed with a 5 × 200 mm column packed with Nucleosil C₁₈ (10 µm) with methanol-water (1 : 1) as mobile phase. The flow rate was 1.0 ml/min. A sample loop containing 20 µl was used. The compounds were detected with a UV-detector at 275 nm. The retention times were 2.9 min for hydroquinone and 3.7 min for p-benzoquinone.

Immobilization of peroxidase. Peroxidase was immobilized on Sepharose 4B using cyanogen bromide activation according to Kohn and Wilchek (1982). 100 mg CNBr was used to activate 10 g wet gel. 60 mg horse radish peroxidase (Sigma, type I) was dissolved in 7.5 ml 0.1 M carbonate buffer (pH 8.5) and was mixed with the activated gel. The mixture was incubated on a rocking table at 4°C over night. The gel was treated with 10 ml 0.5 M ethanolamine (pH 8.8) for 1 h at room temperature and was then washed with 0.1 M carbonate buffer (pH 8.5), 1 M NaCl and 0.1 M succinate buffer (pH 5.0). 46% of the enzyme was coupled to the gel. Accordingly, the gel contained 2.8 mg enzyme per g wet gel.

Determination of hydrogen peroxide. The concentration of the hydrogen peroxide solution (30% Perhydrol, Merck) was determined by titration with potassium permanganate. The permanganate solution was standardized using sodium oxalate (dried at 110°C) as a primary standard.

Results and discussion

The conversion of glycerol to dihydroxyacetone by *Gluconobacter oxydans* with oxygen as electron acceptor has a pH-optimum at 5 (Holst et al. 1982; Adlercreutz et al. in prep.). To check if pH 5 is also optimal when p-benzoquinone is used as electron acceptor, experiments were carried out at pH 4, 5, and 6. In all experiments 0.1 M succinate buffers containing 10 mM CaCl_2 were used. The highest

production rate was obtained at pH 5. The rate at pH 4 was 83% and at pH 6, 58% of the rate at pH 5.

To investigate the stoichiometry of the reaction an experiment was done with p-benzoquinone present in excess. The initial concentrations were 15 mM glycerol and 30 mM p-benzoquinone. After completion of the reaction the solution was analyzed. The concentrations of the products were 15.2 mM dihydroxyacetone and 15.1 mM hydroquinone while the final concentration of p-benzoquinone was 16.2 mM. It was assumed that all the glycerol had been consumed. This experiment suggests that the formula in Fig. 1b is correct.

Influence of concentration of p-benzoquinone

When free cells were used to produce dihydroxyacetone with p-benzoquinone as electron acceptor the production rate was independent of the concentration of p-benzoquinone in the 20–80 mM range (Fig. 2). When 100 mM p-benzoquinone was used the production rate was somewhat lower.

When immobilized cells were used for the conversion in Fig. 1b the production rate increased with increasing concentration of p-benzoquinone up to 60 mM (Fig. 2). A further increase to 100 mM did not increase the production rate further. The maximal productivity of the immobilized cells was about 40% of that of the free cells.

In order to further study the influence of the concentration of p-benzoquinone on the reaction rate of free cells a second series of experiments was performed using lower substrate concentrations. When these low concentrations were used the HPLC-analysis of dihydroxyacetone was not sensitive enough. Instead, the concentration of the other product, hydroquinone, was measured. This compound has a much higher absorbance which means that smaller amounts can be detected. All tests have shown that dihydroxyacetone and hydroquinone are formed in equimolar amounts.

The curve in Fig. 3 resembles Michaelis-Menten kinetics at concentrations above 3 mM. At lower concentrations the curve is sigmoidal. Apparently Michaelis-Menten kinetics is not the perfect approximation in the range below 3 mM. The concentration of p-benzoquinone that gave half of the maximal reaction rate was 3–4 mM.

Comparison of productivities obtained with oxygen and p-benzoquinone.

Identical *Gluconobacter oxydans* preparations have been used with both oxygen and p-benzoquinone as

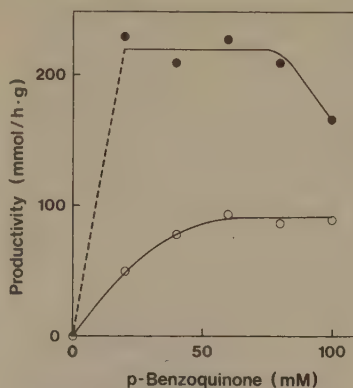


Fig. 2. Production of dihydroxyacetone by *Gluconobacter oxydans* cells as a function of the concentration of p-benzoquinone. The glycerol concentration was 0.25 M. Experiments were done with free cells (●) and cells immobilized in calcium alginate (○) (cell density: 28 g/l). The productivity was calculated from measurements of the concentration of dihydroxyacetone after 5 and 15 min incubation. In the experiments with free cells, 5 mg (dry weight) was used while 12 mg cells was used in the experiments with immobilized cells

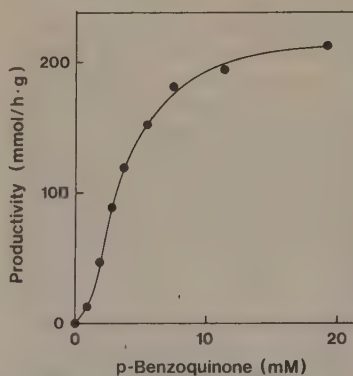


Fig. 3. Production of dihydroxyacetone by free *Gluconobacter oxydans* cells as a function of the concentration of p-benzoquinone. The glycerol concentration was 0.50 M. The productivity was calculated from measurements of the concentration of hydroquinone after 5 and 15 min incubation. The amount of bacteria was 0.25 mg (dry weight) in each experiment

electron acceptors. The results are shown in Table 1. When free cells were used the productivity obtained with p-benzoquinone was more than four times that obtained with oxygen as electron acceptor. In these experiments the concentration of the electron acceptor was not a limiting factor. The cells were saturated with both glycerol and electron acceptor. It is thus evident that the reaction involving p-benzoquinone has a higher maximal rate than the reaction involving oxygen.

When immobilized cells are used p-benzoquinone gives another advantage compared to oxygen; its

Table 1. Productivity of *Gluconobacter oxydans* cells with oxygen (Adlercreutz et al. in preparation) or p-benzoquinone as electron acceptor. Productivities are expressed as mmoles of dihydroxyacetone produced per hour and gram of cells (dry weight). The cells were free or immobilized in calcium alginate gel (cell density: 28 g/l; particle diameter: 2,2 mm). Oxygen was used either as air saturated or oxygen saturated solution. The concentration of p-benzoquinone was 40 mM. The temperature was 30° C

	Productivity (mmol/h · g)		
	p-benzoquinone	Oxygen	
Free cells	350	Air saturated	75
		Oxygen saturated	75
Immobilized cells	109	Air saturated	6.4
		Oxygen saturated	18

solubility is much higher. A buffer solution saturated with oxygen contains just above 1 mM oxygen, while the same buffer solution can dissolve about 100 mM p-benzoquinone. This means that diffusion of oxygen will be the rate limiting factor when immobilized cells are used for aerobic processes.

Because a high bulk phase concentration of p-benzoquinone can be used, the diffusion of this substance to the cells in the beads will be relatively rapid. This means that the differences in productivity with the different electron acceptors will be even more pronounced when immobilized cells are used. When identical cell preparations were used the productivity with p-benzoquinone was 17 times that obtained with air saturation and six times that obtained with oxygen saturation of the substrate solution (Table 1).

It is also interesting to note that the productivity obtained with the standard immobilized preparation with p-benzoquinone as electron acceptor was considerably higher than that obtained with free cells and oxygen as electron acceptor.

Influence of cell density

To investigate how the cell density in the beads influence the productivity four different preparations were made. The cell density was between 4 and 28 g/l. The rate of dihydroxyacetone production was measured under identical conditions. As expected, the efficiency decreased with increasing cell density (Fig. 4). This means that diffusion (of p-benzoquinone) through the gel matrix is a rate limiting factor. However, diffusion limitations were not very pronounced in the 4 g/l preparation. When this was used the rate (183 mmol/g · h) approached the rate achieved with free cells (227 mmol/g · h).

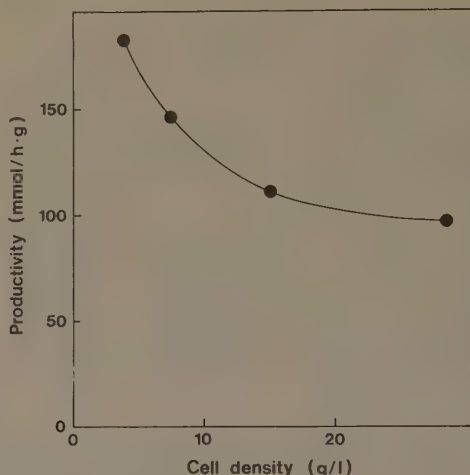


Fig. 4. The productivity of immobilized *Gluconobacter oxydans* cells as a function of the cell density in the alginate beads. Gel beads from the different preparations were added to substrate solutions to get the concentrations of 0.25 M glycerol and 60 mM p-benzoquinone. In each experiment the gel contained 12 mg cells. Samples were taken every 5th min

Product inhibition

During the oxidation of glycerol to dihydroxyacetone p-benzoquinone is converted to hydroquinone. If the concentrations of p-benzoquinone and hydroquinone are high enough quinhydrone is formed. Quinhydrone is a 1:1-complex formed from p-benzoquinone and hydroquinone. The complex is only slightly soluble in water. At higher concentrations quinhydrone precipitates as black crystals. In the experiments in this study quinhydrone was usually formed after some time if the initial concentration of p-benzoquinone was above 50 mM. The precipitation is readily reversible. When the bioconversions in this study approached completeness and most of the p-benzoquinone had been converted to hydroquinone the precipitate dissolved. If hydroquinone is present in excess the concentration of free p-benzoquinone will be low and the production rate of the immobilized cells will then also be low.

To investigate this inhibiting effect of hydroquinone a series of experiments were done where different amounts of hydroquinone were present in the substrate solution from the beginning of the reaction. It was shown that the reaction rate decreases with increasing concentration of hydroquinone (Table 2).

Experiments were also performed with more p-benzoquinone than could be dissolved in the buffer. For example, p-benzoquinone corresponding to a final concentration of 200 mM was added. In this

Table 2. Inhibition by hydroquinone of the production of dihydroxyacetone by immobilized *Gluconobacter oxydans* cells. The productivity was measured in solutions containing 0.25 M glycerol and 60 mM p-benzoquinone. 14 mg cells was used in each experiment and the cell density in the gel was 28 g/l. The concentration of dihydroxyacetone was measured after 5 and 15 min of reaction. The productivity in the solution without any hydroquinone was set at 100%

Hydroquinone (mM)	Relative productivity (%)
0	100
50	69
100	30
200	15

experiment a lot of quinhydrone was formed but eventually the precipitate dissolved. It was found that all p-benzoquinone could be converted to hydroquinone and a corresponding amount of dihydroxyacetone was formed. However, the reaction was somewhat slower than in experiments with p-benzoquinone concentrations around 60 mM.

One disadvantage with the use of high concentrations of p-benzoquinone is that quinhydrone tends to form a shell around the gel beads. This shell reduces the reaction rate because the substrates must diffuse through it. Another disadvantage is that the alginate beads tend to be less stable when a lot of quinhydrone is formed, perhaps because quinhydrone crystals are formed inside the beads and disrupt their structure.

Operational stability

If p-benzoquinone is to be used as an oxygen substitute in industrial processes it is essential that the enzymes or cells used to carry out the reaction are efficient for a long period of time. Inactivation must not be too fast under operation conditions.

The operational stability was tested in an 8-day experiment. In the first batch a dihydroxyacetone concentration of 23 mM was obtained (Fig. 5). As the initial concentration of p-benzoquinone in the substrate solution was 30 mM, the maximal theoretical product concentration was 30 mM. p-Benzoquinone was present in the substrate solution even at the end of each incubation period, and the cells produced dihydroxyacetone continuously for 8 days except for the short pauses (~5 min) when the substrate solution was changed. The activity decreased a little for each successive batch of substrate, but considerable activity was left at the end of the experiment.

As a comparison, one experiment was performed where oxygen was used as electron acceptor. The immobilized cells produced dihydroxyacetone contin-

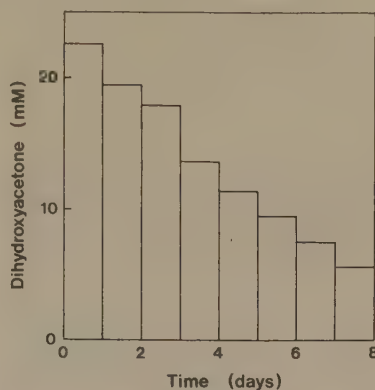


Fig. 5. Operational stability of immobilized *Gluconobacter oxydans* cells converting glycerol to dihydroxyacetone with p-benzoquinone as electron acceptor. The gel contained 0.35 mg cells and had the cell density 28 g/l. The substrate solution contained 0.25 M glycerol and 30 mM p-benzoquinone. One 15 ml batch of substrate solution was used for each 24-h period. The concentration of dihydroxyacetone formed in each successive batch was measured

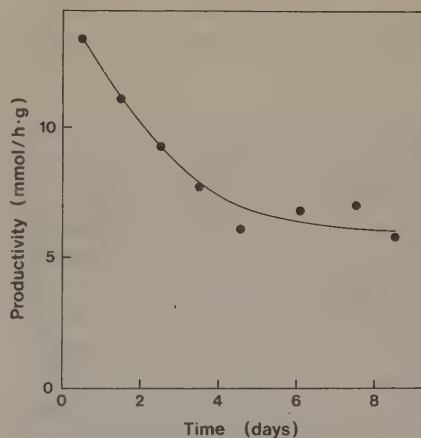


Fig. 6. Operational stability of *Gluconobacter oxydans* in an oxygen saturated solution. Alginate gel (cell density: 28 g/l) containing 24 mg immobilized bacteria was used to produce dihydroxyacetone. The reactor volume was 105 ml and the glycerol concentration was 0.25 M. The solution was gently stirred. Oxygen was led through a flask with water to the reactor. The oxygen pressure of the solution was continuously measured with a polarographic oxygen electrode. The oxygen flow was adjusted to get $\geq 95\%$ oxygen saturation. The temperature was 22°C. The medium was exchanged at intervals and the amount of dihydroxyacetone produced was measured

uously for 9 days in a solution that was almost ($\geq 95\%$) saturated with oxygen. During the first 4–5 days the productivity decreased rather rapidly, but after day 5 the productivity was fairly constant (Fig. 6). The results presented in Figs. 5 and 6 cannot be directly compared. In Fig. 5 the concentration of

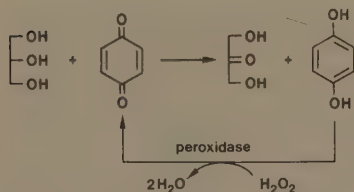


Fig. 7. Formulas for production of dihydroxyacetone with regeneration of p-benzoquinone

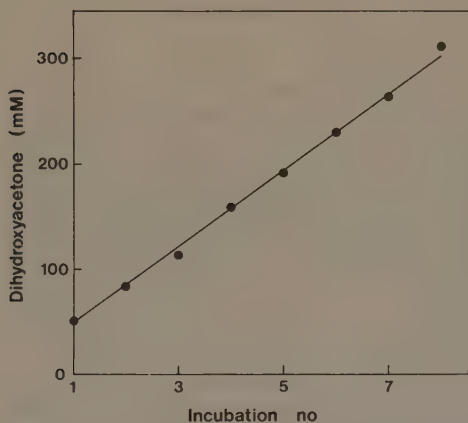


Fig. 8. Production of dihydroxyacetone with regeneration of the electron acceptor p-benzoquinone. *Gluconobacter oxydans* cells (65 mg dry weight) immobilized in calcium alginate gel (cell density: 28 g/l) were put into a tube containing 25 ml substrate solution (0.50 M glycerol and 50 mM p-benzoquinone in succinate buffer). The tube was closed with a screw-cap and was incubated on a rocking table until all p-benzoquinone had been converted to hydroquinone (45 min; the reaction could be followed with HPLC-analysis). The alginate gel was filtered off. A sample (0.4 ml) was taken from the solution for analysis of dihydroxyacetone, while the rest was poured into a beaker together with immobilized peroxidase (0.8 mg enzyme). Hydrogen peroxide was added to get a final concentration of 40 mM. The mixture was magnetically stirred for 8 min. During this time p-benzoquinone was regenerated to get a concentration of about 40 mM. Then the immobilized enzyme was filtered off and the solution was poured back into the tube with the immobilized cells. After another 45 min incubation the next sample was taken for analysis of dihydroxyacetone. Then p-benzoquinone was regenerated once again. In all, eight incubations with the immobilized cells were carried out and p-benzoquinone was regenerated seven times. The concentration of dihydroxyacetone after the successive incubations is shown

p-benzoquinone varied with time while the oxygen concentration was constant in Fig. 6. However, it is possible to calculate the activity of the cells during the p-benzoquinone experiment. The activity during the 8th day was 10 mmol/h · g. The mean concentration of p-benzoquinone during the 8th day was 27 mM. At this concentration the activity of cells directly after immobilization is about 60 mmol/h · g (Fig. 2). While

the activity in the p-benzoquinone experiment decreased from 60 to 10 mmol/h · g, the activity in the oxygen experiment decreased from 14 to 6 mmol/h · g (Fig. 6). Apparently the decrease was a little faster when p-benzoquinone was used but the productivity was higher in the p-benzoquinone experiment even during the last day of the experiments.

Considering the very high initial productivity (compared to when oxygen is used) and the fairly good operational stability, p-benzoquinone seems to be a realistic alternative as an oxygen substitute in microbial oxidation reactions.

Regeneration of p-benzoquinone

When p-benzoquinone is used as an electron acceptor hydroquinone is formed as a byproduct. In an industrial process it would be beneficial to use this byproduct. One obvious alternative is to oxidize hydroquinone to p-benzoquinone which can be re-used. We have used hydrogen peroxide and immobilized peroxidase to carry out this reaction (Fig. 7). As hydrogen peroxide is harmful to the cells (Holst et al. 1982), this reaction was carried out in the absence of cells. It was possible to recycle p-benzoquinone several times. The efficiency of dihydroxyacetone production did not decrease during 8 successive cycles (Fig. 8).

When carried out like this, glycerol and hydrogen peroxide are consumed in the process and dihydroxyacetone is formed. The yields are quite good. The yield with respect to hydrogen peroxide was about 90%.

General discussion

In the system studied, p-benzoquinone is a more efficient electron acceptor than oxygen. This effect can be divided in two parts. Firstly, p-benzoquinone gives a higher maximal rate (the rate when the cells are saturated with the substrates). This effect is advantageous both when free and immobilized cells are used. Secondly, the solubility of p-benzoquinone is much higher than that of oxygen in the water solutions used. This is a major advantage when immobilized cells are used. The high bulk concentration gives a high driving force for diffusion from the bulk phase to the cells in the matrix.

There are also factors that favour the use of oxygen. The cells have a higher affinity for oxygen than for p-benzoquinone. K_m for oxygen is in the μM -range (Enfors and Mattiasson 1983) while " K_m " for p-benzoquinone is in the mM-range. Oxygen is also a smaller molecule that diffuses more rapidly

through the gel matrix than p-benzoquinone. However, these factors are apparently less important than the factors favouring the use of p-benzoquinone.

The enzyme or enzymes responsible for the oxidation of glycerol in *Gluconobacter oxydans* cells have not been thoroughly characterized. It is not completely clear how p-benzoquinone works in this reaction. One possible mechanism is that p-benzoquinone directly oxidizes the reduced form of the enzyme. Many oxidative enzymes can use artificial electron acceptors. For example mannitol dehydrogenase (E.C. 1.1.2.2) from *Aerobacter suboxydans* and D-lactate dehydrogenase (E.C. 1.1.2.4) from aerobic yeast can use 2,6-dichlorophenol indophenol and ferricyanide as electron acceptors (Barman 1969).

One partially purified enzyme from *Gluconobacter oxydans* (ATCC 621) could use artificial electron acceptors such as ferricyanide and 2,6-dichlorophenol indophenol when mannitol was oxidized (Arcus and Edson 1956). This enzyme could also oxidize glycerol. Since the enzyme preparation also contained cytochromes it is not sure that the artificial electron acceptors work directly on the glycerol oxidizing enzyme.

Normally the glycerol oxidizing enzyme is probably re-oxidized by an electron transport chain containing ubiquinone and cytochromes (Daniel 1970). One possible mechanism is that p-benzoquinone oxidizes some component of this chain, so that part of the chain is in operation but p-benzoquinone is used as terminal electron acceptor instead of oxygen.

The fact that p-benzoquinone gives a higher maximal rate than oxygen shows that some rate limiting step in the normal oxidation reaction is avoided by the use of p-benzoquinone. One possible explanation is that some reaction in the electron transport chain is rate limiting and that p-benzoquinone diverts the electrons before this reaction.

In this study it is shown that p-benzoquinone can be re-used several times. The regeneration was carried out batchwise. A future development is the continuous regeneration of p-benzoquinone.

In a hypothetical industrial process the main reactor contains immobilized *Gluconobacter oxydans* cells that oxidize glycerol to dihydroxyacetone with p-benzoquinone as electron acceptor. The production medium (free of cells) is continuously recycled through a column containing immobilized peroxi-

dase. Before this column hydrogen peroxide is added. In the main reactor p-benzoquinone is reduced to hydroquinone which is re-oxidized in the immobilized enzyme column.

The oxidation of glycerol to dihydroxyacetone should just be considered as an example of a microbial oxidation reaction. Hopefully p-benzoquinone and similar artificial electron acceptors can be used to increase the efficiency of many similar bioconversions.

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References

- Adlercreutz P, Holst O, Mattiasson B (in preparation)
- Adlercreutz P, Mattiasson B (1982) Oxygen supply to immobilized cells: 3. Oxygen supply by hemoglobin or emulsions of perfluorochemicals. *Eur J Appl Microbiol Biotechnol* 16: 165–170
- Adlercreutz P, Holst O, Mattiasson B (1982) Oxygen supply to immobilized cells: 2. Studies on a coimmobilized algae-bacteria preparation with in situ oxygen generation. *Enzyme Microbiol Technol* 4: 395–400
- Alberti BN, Klivanov AM (1982) Preparative production of hydroquinone from benzoquinone catalysed by immobilized D-glucose oxidase. *Enzyme Microbiol Technol* 4: 47–49
- Arcus AC, Edson NL (1956) Polyol dehydrogenases. 2. The polyol dehydrogenases of *Acetobacter suboxydans* and *Candida utilis*. *Biochem J* 64: 385–394
- Barman TE (1969) *Enzyme Handbook*, vol 1. Springer, Berlin Heidelberg
- Buckland BC, Dunnill P, Lilly MD (1975) The enzymatic transformation of waterinsoluble reactants in nonaqueous solvents. Conversion of cholesterol to cholest-4-ene-3-one by a *Nocardia* sp. *Biotechnol Bioeng* 17: 815–826
- Daniel RM (1970) The electron transport system of *Acetobacter suboxydans* with particular reference to cytochrome o. *Biochim Biophys Acta* 216: 328–341
- Enfors SO, Mattiasson B (1983) Oxygenation of processes involving immobilized cells. In: Mattiasson B (ed) *Immobilized cells and organelles*. CRC-Press, London pp 41–60
- Holst O, Enfors SO, Mattiasson B (1982) Oxygenation of immobilized cells using hydrogen-peroxide; a model study of *Gluconobacter oxydans* converting glycerol to dihydroxyacetone. *Eur J Appl Microbiol Biotechnol* 14: 64–68
- Kohn J, Wilchek M (1982) A new approach (cyano-transfer) for cyanogen bromide activation of sepharose at neutral pH, which yields activated resins, free of interfering nitrogen derivatives. *Biochem Biophys Res Commun* 107: 878–884

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Oxygen supply to immobilized cells:

5. Theoretical calculations and experimental data for the oxidation of glycerol by immobilized Gluconobacter oxydans cells with oxygen or p-benzoquinone as the electron acceptor.

Patrick Adlercreutz

Department of Biotechnology, Chemical Center,
University of Lund, P.O.Box 124, S-221 00 Lund, Sweden

Running title: Oxygen supply to immobilized cells

Key words: immobilized cells, oxygen transfer, artificial electron acceptors, p-benzoquinone, Gluconobacter oxydans.

SUMMARY

Theoretical calculations of reaction kinetics were done for one-step reactions catalysed by cells immobilized in spherical beads. The reactions catalysed by free cells were assumed to obey Michaelis-Menten kinetics for a one substrate reaction. Both external (outside the beads) and internal (inside the beads) mass transfer of the substrate were considered for the immobilized preparations. The theoretical calculations were compared with experimental data for the oxidation of glycerol to dihydroxyacetone by Gluconobacter oxydans cells immobilized in calcium alginate gel. Glycerol was present in excess so that the reaction rate was limited by oxygen. The correlation between experimental data and theoretical calculations was quite good. The calculations showed how the overall effectiveness factor was influenced by, for example, the particle size and the cell density in the beads. In most cases the reaction rate was mainly limited by internal mass transfer of the substrate (oxygen). As shown previously, p-benzoquinone can replace oxygen as the electron acceptor in this reaction. The same equations for reaction kinetics and mass transfer were used with p-benzoquinone as the rate limiting substrate. Parameters such as diffusivity, maximal reaction rate and K were, of course, different. In this case also, the correlation between the model and the experimental results was quite good. Much higher production rates were obtained with p-benzoquinone as the electron acceptor compared to when oxygen was used. The reasons for this fact were that p-benzoquinone gave a higher maximal reaction rate for free cells and that the solubility of p-benzoquinone was higher than for oxygen. Different methods of increasing the rate of microbial oxidation reactions are discussed.

INTRODUCTION

When microbial cells are to be used in biotechnological processes, immobilization of the cells is often beneficial. One main advantage is that the immobilized cells are easily separated from the reaction products. However, immobilization also creates problems; for example, the mass transfer resistance is increased for the substrates and products. One case where this effect is of great importance is in the use of oxygen demanding cells. When aerobic cells with a high catalytic activity are used, oxygen supply is often a critical point.

Immobilization makes these problems even more severe. In order to handle these problems, it is necessary to know the importance of the different factors governing the reaction rate. A review article about the correlation of experimental and theoretical data for systems with immobilized biocatalysts was recently written by Kasche¹.

This report concerns cells immobilized in spherical beads. Using computer calculations it is shown how the effectiveness factor of an immobilized preparation is dependent on different factors such as the cell density and the particle size. The theoretical calculations are compared with experimental data.

Similar calculations have been performed by Hiemstra et al. who studied the oxidation of methanol by Hansenula polymorpha cells immobilized in barium alginate gel². Sato and Toda have studied the oxygen uptake rate of Candida lipolytica cells immobilized in an agar gel³. These authors also measured the diffusivity of oxygen in the gel and performed theoretical calculations of the effectiveness factor for immobilized cell preparations.

In this study Gluconobacter oxydans (ATCC 621) was used as an example of an oxygen demanding organism. These bacteria oxidize glycerol to dihydroxyacetone. Normally oxygen is used as an electron acceptor (Fig 1a). The properties of G. oxydans immobilized in calcium alginate gel have been thoroughly investigated⁴. Recently it was shown that oxygen can be replaced by the artificial electron acceptor p-benzoquinone⁵ (Fig 1b). In this report calculations are performed for both electron acceptors.

MATERIALS AND METHODS

Materials

Sodium alginate was purchased from Sigma (type IV, lot no 111F-0049) and p-benzoquinone (zur Synthese) from Merck.

Organism

Gluconobacter oxydans (ATCC 621) was grown as previously described⁶.

Immobilization

The cells were immobilized in calcium alginate gel as previously described⁵. Preparations with varying particle sizes and cell densities were made. The various particle sizes were obtained by extruding the alginate cell mixture through needles with varying diameters during immobilization. The diameters of the beads were measured microscopically after equilibration in the buffer solution used for the kinetic experiments. The cell density was expressed as kg of cells (dry weight) per m³ of gel phase. The density of the gel particles when equilibrated with the reactant solution was 1027 kg/m³.

Experimental set up

The reactor was designed for measuring the rate of oxygen consumption by immobilized cells. It was a 115 ml glass vessel which could be closed with a rubber stopper. A polarographic oxygen electrode (Radio-meter) was fitted in the rubber stopper. The reactor was placed in a water bath which was thermostated to 30°C. A magnet was fixed on a wire through the stopper so that the solution could be stirred without any direct contact between the magnet and the reactor walls. This was done to avoid deterioration of the gel beads. The stirring rate was 425 rpm in all experiments. Increasing the stirring rate further did not increase the reaction rate.

Kinetic experiments

The buffer solution (0.1 M succinate buffer containing 10 mM CaCl_2 , pH 5.0) was poured into the reactor where it was thermostated and saturated with oxygen. The cells (free or immobilized) were added and the rubber stopper was put on. No gas bubbles were allowed in the reactor. Experiments to determine $K(\text{O}_2)$ used 20 mg of free cells while 2.5-10 mg of cells were used to determine $k_2(\text{O}_2)$. In the experiments with immobilized cells, 25 mg of cells were used. The reaction was initiated by the addition of glycerol through a hypodermic needle. The glycerol concentration in the reactor was 0.25 M. The density of the reactant solution was 1009 kg/m³. The oxygen concentration was measured continuously with the oxygen electrode. Using the diagram of the oxygen concentration as a function of time, the reaction rate was determined from the slope of the curve (not shown). The reaction rate was determined for 5 to 6 oxygen concentrations in each experiment. A normal experiment lasted about 40 min. The influence of the electrode response time was neglected.

The same reactor was used for the experiments using p-benzoquinone as the electron acceptor except that the oxygen electrode was replaced by a small rubber stopper. The glycerol concentration used was 0.50 M. In order to follow this reaction, the amount of hydroquinone formed was measured with reversed phase HPLC⁵. In the experiments with free cells, 4 mg of cells were used while 14 mg of cells were used in the experiments with immobilized cells. Samples were taken with hypodermic syringes at 5 or 8 minute intervals depending on the reaction rate.

CALCULATION PROCEDURES

Problem description

In the idealized system used, the cells were immobilized in perfectly spherical gel beads. All beads were assumed to have the same radii and the cells were assumed to be uniformly distributed in the beads. The reaction studied was the oxidation of glycerol to dihydroxyacetone as shown in figure 1. Oxygen or p-benzoquinone was used as the electron acceptor. Glycerol was always present in excess, so the reaction rate was limited by the concentration of the electron acceptor. The electron acceptor is referred to as the substrate in the following text. The dependence of the reaction rate of free cells on the substrate concentration was assumed to obey Michaelis-Menten kinetics. Other assumptions in the model were that the system was at steady state and that the presence of reaction products did not affect the reaction rate.

The aim of the theoretical part of this study was to calculate the productivity of immobilized cell preparations under specified conditions. Both external and internal mass transfer of the substrate were considered.

Basic equations

As an approximation, Michaelis-Menten kinetics were used to describe the reaction kinetics of free G. oxydans cells:

$$v_{\text{free}} = k_2 \cdot \rho \cdot \frac{c_{\text{bs}}}{c_{\text{bs}} + K} \quad (1)$$

The constants k_2 and K were determined experimentally for each substrate by experiments with free cells.

If the cells are immobilized in gel beads, the reaction rate is often limited by the mass transfer of the substrates and products. In the present case, the reaction rate was limited by transfer of the substrate. The local reaction rate obtained in a small section of a bead is determined by the local substrate concentration, c_{is} .

$$v_{\text{loc}} = k_2 \cdot \rho \cdot \frac{c_{\text{is}}}{c_{\text{is}} + K} \quad (2)$$

The c_{is} varies with the distance to the surface of the bead. As c_{is} is less than c_{bs} the observed reaction rate is lower than the rate obtained with free cells. The ratio of the actual reaction rate to the rate that would be obtained in the absence of diffusion resistances is called the overall effectiveness factor, η_0 .

$$\eta_0 = \frac{v_{\text{obs}}}{v_{\text{free}}} \quad (3)$$

$$v_{\text{obs}} = -D_{\text{is}} \cdot \left(\frac{dc_{\text{is}}}{dr} \right)_{r=r_0} \cdot \frac{3}{r_0} \quad (4)$$

In this study, both internal (inside the beads) and external (outside the beads) mass transfer were considered. For external mass transfer, the following equation was used:

$$k_c \cdot (c_{bs} - c_{os}) = -D_{is} \cdot \left(\frac{dc_{is}}{dr} \right)_{r=r_0} \quad (5)$$

The equations for mass transfer and for the reaction kinetics can be combined. The following equation can be applied for spherical particles:

$$D_{is} \cdot \left(\frac{d^2 c_{is}}{dr^2} + \frac{2}{r} \frac{dc_{is}}{dr} \right) = \frac{k_2 \cdot \rho \cdot c_{is}}{K + c_{is}} \quad (6)$$

The boundary conditions which apply are:

$$r = 0 \quad \frac{dc_{is}}{dr} = 0 \quad (7)$$

$$r = r_0 \quad c_{is} = c_{os} \quad (8)$$

Equations like this one cannot be solved analytically. Several numerical methods have been used to solve equation (6). In this study the calculation procedure published by Fink et al⁷ was used. In these calculations, the Damköhler number, β , is used to describe mass transfer in the gel phase, and the Sherwood number, α , is used to describe external mass transfer.

$$\beta = \frac{k_2 \cdot \rho \cdot r_0^2}{D_{is} \cdot K} \quad (9)$$

$$\alpha = \frac{k_c \cdot r_0}{D_{is}} \quad (10)$$

The overall effectiveness factor, η_0 , is determined by c_{bs} , the substrate concentration in the bulk liquid phase, α and β .

Computer calculations

All calculations were performed on a UNIVAC 1100 computer. The method used was that of Fink et al.⁷. The differential equation was transformed and then solved numerically using a Runge-Kutta integration routine. The calculations were done in double precision. Normally, a grid spacing of $\Delta x=0.001$ was used.

Calculations were done for different α and β values. The results were expressed as curves with the overall effectiveness factor, η_0 , as a function of the substrate concentration in the bulk liquid phase. In this way, it was easy to compare the theoretical curves with the experimental ones. Another alternative would be to plot the overall effectiveness factor as a function of the Thiele modulus.

Determinations of α and β values

The results of the experiments were expressed as curves with the overall effectiveness factor, η_0 , as a function of the substrate concentration in the bulk liquid phase, c_{bs} . These curves were compared with theoretical curves obtained with different α and β values. The α and β values were varied until the best correlation was found for each immobilized preparation. In order to start this procedure, the approximation was made that external mass transfer resistance could be neglected ($\alpha = \infty$), and the β values were calculated. Then α values were calculated. Using these α values, better β values

were obtained. After a few such cycles constant values of α and β were obtained.

From each of the β values D_{js} was calculated using equation (9). The mean value of these D_{js} calculations was used in the rest of the calculations. Once D_{js} was known, β could be calculated for hypothetical immobilized preparations having different cell densities and bead radii.

Calculations for hypothetical immobilized preparations

One goal of this investigation was to make it possible to theoretically calculate the overall effectiveness factor for hypothetical immobilized preparations. To do this, one must be able to calculate α and β values from the data normally known about a preparation such as the cell density and the particle size. Of the different factors in β , k_2 and K were determined with free cells. D_{js} was determined as shown above. The ρ and r_0 values varied for the different immobilized preparations.

The Sherwood number depends on the particle size. Approximations of the Sherwood number are often expressed as shown below ^{8,9} :

$$\frac{k_c \cdot d_p}{D} = 2.0 + \text{const.} \cdot \text{Re}^{\frac{1}{2}} \cdot \text{Sc}^{\frac{1}{3}} \quad (11)$$

As an approximation it was assumed that the particles in the reactor reached terminal velocity. For this case Harriott⁸ used eq (11) and plotted k_c as a function of d_p . In the range $0.2 \text{ mm} < d_p < 10 \text{ mm}$, k_c was almost independent of d_p . This is also found experimental-

ly⁸. Accordingly k_c was considered constant in the calculations. As D_{is} was also considered constant, α was proportional to r_0 . The experimental α values obtained with different immobilized preparations were plotted against r_0 and a straight line was drawn. The slope of the line was 39000. Therefore, the following equation was used to calculate α values for immobilized preparations with oxygen as the substrate:

$$\alpha = 39000 \cdot r_0 \quad (12)$$

Determination of α and β values in experiments with p-benzoquinone

In the experiments with p-benzoquinone as the electron acceptor, the influence of external mass transfer on the overall effectiveness factor was rather small. Therefore, α and β values were not determined as when oxygen was used as electron acceptor. Instead, α values were calculated using data from the oxygen experiments. To express α as a function of r_0 , both $k_c(p-b)$ and $D_{is}(p-b)$ must be known (see eq (10)). From the oxygen experiments, $k_c(O_2)$ was calculated ($k_c(O_2) = 8.23 \cdot 10^{-5}$ m/s). The $k_c(p-b)$ value was calculated using the following approximate equation⁸:

$$k_c = \text{const.} \cdot D^{\frac{2}{3}} \quad (13)$$

Diffusivities for oxygen and p-benzoquinone in water (at 25°C) were found in the literature: $D(O_2) = 2.41 \cdot 10^{-9}$ m²/s⁹ and $D(p-b) = 1.03 \cdot 10^{-9}$ m²/s¹⁰. Using these values, $k_c(p-b)$ was calculated ($k_c(p-b) = 4.67 \cdot 10^{-5}$ m/s). The $D_{is}(p-b)$ value was

not known. As a first approximation $D_{is}(p-b)$ was calculated using the following equation:

$$\frac{D_{is}(p-b)}{D(p-b)} = \frac{D_{is}(O_2)}{D(O_2)} \quad (14)$$

This calculation gave the result $D_{is}(p-b) = 9.02 \cdot 10^{-10} \text{ m}^2/\text{s}$. Then α values for the experiments with p-benzoquinone were calculated and β values were then calculated for each experiment. Using 7 different immobilized preparations and 2 different substrate concentrations, 14 determinations of $D_{is}(p-b)$ were done. The mean value was then used in eq (10) to calculate new α values which gave new β values and so on. From the final β values, $D_{is}(p-b)$ was calculated. The mean value, $7.2 \cdot 10^{-10} \text{ m}^2/\text{s}$, was used in the rest of the calculations. For α , the following equation was obtained:

$$\alpha = 65000 \cdot r_0 \quad (15)$$

RESULTS AND DISCUSSION

Determination of kinetic constants

The constants k_2 and K in equation (1) were determined for each substrate by measurements on free cells. For oxygen, k_2 was easily determined from the oxygen uptake rate at high oxygen concentrations ($k_2(O_2) = 1.036 \cdot 10^{-5} \text{ kmol/s} \cdot \text{kg}$). Measurements of K were more difficult because the value of this constant was in the μM range and the equipment used did not allow any detailed kinetic studies in this range. However, it was possible to determine the oxygen concentration which gave half the maximal reaction rate ($K(O_2) = 8.6 \cdot 10^{-6} \text{ kmol/m}^3$).

The reaction rate as a function of the concentration of p-benzoquinone has already been studied⁵. Minor deviations from Michaelis-Menten kinetics were apparent, but Michaelis-Menten kinetics were still a reasonably good approximation. The best correlation with the experimental data was obtained with $K(\text{p-b}) = 3.5 \cdot 10^{-3} \text{ kmol/m}^3$. The $k_2(\text{p-b})$ value was determined to be $1.069 \cdot 10^{-4} \text{ kmol p-b/s} \cdot \text{kg}$ at 30°C .

Introducing calculations

A certain immobilized preparation is characterized by its Damköhler number, β . The β value is determined by the cell density and the particle size, as well as the constants D_{jS} , k_2 and K (equation (9)). The overall effectiveness factor, η_0 , of a certain preparation varies with the bulk substrate concentration. If the substrate concentration is sufficiently high, all the immobilized cells are saturated with the substrate and η_0 is 1.0. However, when the substrate is oxygen this is seldom the case. Only the cells near the

surface of the beads have enough oxygen to carry out the reaction at any appreciable rate and η_0 is less than 1.0. In figure 2, η_0 is given as a function of the bulk oxygen concentration for different β values. The higher the β value, the more pronounced are diffusion limitations.

The β value describes only diffusion inside the beads. However, diffusion through the stagnant liquid film surrounding the beads must also be taken into consideration. This resistance to mass transfer is described by the Sherwood number. Figure 3 shows how the Sherwood number, α , influences the overall effectiveness factor for a given β value. If external mass transfer resistance can be neglected, α approaches infinity. The α value is mainly determined by the motion of the beads in the liquid. Differences in α caused much smaller effects on η_0 than differences in β .

In the range 0.2-1.0 mM oxygen, the shape of the curves is almost exclusively determined by the β values while the α values cause a shift of the curves parallel with the η_0 -axis.

Correlation of experiments and calculations

When the external diffusion limitations were neglected ($\alpha = \infty$), it was not possible to find theoretical curves that corresponded well with the experimental data. But then external mass transfer was also considered and β and α values were determined for each preparation to give the best fit with the experimental data (Table 1). Some curves are shown in Fig 4.

Oxygen diffusivity in the gel

From the β values for each preparation, the diffusivity of oxygen in the gel could be calculated (equation (9)). These values are shown in table 1. The mean value of D_{js} ($2.11 \cdot 10^{-9} \text{ m}^2/\text{s}$) was used in the rest of the calculations. The diffusivity of oxygen in pure water at 25°C is $2.41 \cdot 10^{-9} \text{ m}^2/\text{s}$ ⁹. From this value, the diffusivity at 30°C was calculated using the following equation^{9,11}:

$$D = \text{const.} \cdot \frac{T}{\mu} \quad (16)$$

According to this equation, the diffusivity at 30°C was $2.74 \cdot 10^{-9} \text{ m}^2/\text{s}$. The diffusivity of oxygen in the gel was thus 77 % of the diffusivity in pure water. Hiemstra et al. found that the diffusivity of oxygen in a barium alginate gel was about 25 % of the diffusivity in water². Sato and Toda used a special apparatus to measure the diffusivity of oxygen in an agar gel³. They found that it was 70 % of the diffusivity in water.

Theoretical calculations for different conditions

The determined value of D_{js} was used in the theoretical calculations to test the correlation with the experimental data. Calculations were done for the experimental conditions (r_0 , ρ , $c_{bs} = 0.5 \cdot 10^{-3} \text{ kmol/m}^3$). The α values were calculated according to eq (12). When the calculated overall effectiveness factor, $\eta_{o,\text{calc}}$, was compared with the experimental data, $\eta_{o,\text{exp}}$, the correlation was quite good (Table 1).

Once D_{js} was determined, it was also possible to do theoretical calculations of the overall effectiveness factor for different hypothetical conditions. The results of some example calculations are

shown in figure 5. Of course, one should be careful with extrapolations of this kind. The calculations are based on several assumptions and it is not quite certain that all of these assumptions are valid for the entire range of figure 5. Nevertheless, figure 5 should give a good view of alternative ways of reaching high effectiveness factors. For example, it can be deduced that if beads are made with a radius of 1.0 mm and an overall effectiveness factor of at least 0.20 is wanted, cell densities exceeding 10 kg/m^3 must not be used. In order to reach high overall effectiveness factors with a high cell density, it would be necessary to use very small particles ($r_0=0.1 \text{ mm}$).

The use of Michaelis-Menten kinetics

The reactions catalysed by enzymes or whole cells are often described by Michaelis-Menten kinetics. In this study Michaelis-Menten kinetics was also a fairly good approximation of the concentration dependence of the reaction rate of free G. oxydans cells. Michaelis-Menten kinetics have sometimes been used to describe the kinetics of immobilized cell preparations as well. However, this is a good approximation only if diffusion limitations are of little importance. The more severe the diffusion limitations are, the more deviations from Michaelis-Menten kinetics can be expected.

In the model used in this report, Michaelis-Menten kinetics of the free cells were used in combination with the equations for mass transfer. Both external and internal diffusion were considered. When the theoretical curves were plotted in the double reciprocal way (Lineweaver Burk plot) all the curves bent and met in a point corresponding to $\eta_0=1.0$ (Fig 6). These curves correspond well with

those obtained by Regan et al. from the same type of calculations¹². In limited ranges, the curves are almost straight lines. It is, for example, easy to draw straight lines for the experimental data in figure 6. However, predictions of the reaction rates outside the concentration range studied should not be made by extrapolations of the straight lines.

Concentration profiles of oxygen inside the beads

The concentration profiles inside the beads were calculated. The results of some such calculations are shown in figure 7. When the bulk liquid phase was saturated with air, only the cells in a very thin zone near the surface were active. If pure oxygen was used instead of air, more cells were active but the diffusion limitations were still severe. In the middle of the beads, the oxygen concentration was very low.

Calculations and experiments with p-benzoquinone as the electron acceptor

When the substrate was changed from oxygen to p-benzoquinone, almost all of the parameters in the calculations had to be changed. For example, p-benzoquinone has a higher maximal rate (k_2), a higher K and a lower D_{js} value compared to oxygen's.

Fourteen separate experiments were performed to estimate the diffusivity of p-benzoquinone in the gel. The results are shown in table 2. The mean value ($D_{js} = 7.2 \cdot 10^{-10} \text{ m}^2/\text{s}$) was used in the rest of the calculations.

The diffusivity of p-benzoquinone in pure water is $1.03 \cdot 10^{-9} \text{ m}^2/\text{s}$ at 25°C. Using eq (16) the diffusivity at 30°C was calculated to be $1.17 \cdot 10^{-9} \text{ m}^2/\text{s}$. Thus, the diffusivity of p-benzoquinone in the gel

was 62 % of the diffusivity in pure water. The p-benzoquinone molecule, which is bigger than oxygen, is apparently more retarded by the alginate gel.

As in the oxygen experiments, the calculated value of D_{is} was used to test the correlation of the theoretical and the experimental data. The calculated overall effectiveness factors, $\eta_{o,calc}$, were compared to the experimental data, $\eta_{o,exp}$ (Table 2). In most cases the correlation was quite good.

Theoretical calculations were also done to cover a wide range of particle radii and cell densities. The results are shown in figure 8. In comparison with figure 5, all curves are moved towards higher effectiveness factors. The reason for this is that much higher substrate concentrations are used with p-benzoquinone than with oxygen. As a result, bigger beads can be used when p-benzoquinone is the substrate instead of oxygen. This is an advantage as small beads (0.1-0.2 mm) are difficult to make and also difficult to handle.

In figure 8, a bulk substrate concentration of 40 mM was used. At higher concentrations of p-benzoquinone, complications can occur caused by the formation of quinhydrone, which is a 1:1-complex formed from p-benzoquinone and hydroquinone. The formation of quinhydrone can affect the reaction rate but, as shown before, the reaction rate increases with the concentration of p-benzoquinone up to at least 60 mM⁵.

Just as in the case for oxygen, the concentration profile of p-benzoquinone in the beads was calculated (Fig 9). As expected the substrate concentrations are much higher than for oxygen.

GENERAL DISCUSSION

Immobilized G. oxydans cells have been used to evaluate several methods of increasing oxygen supply to immobilized cells. These methods increase the production rate by different mechanisms. When algae were co-immobilized with the bacteria, oxygen was generated inside the gel beads¹³. Virtually all the oxygen formed was utilized by the bacteria. Another way to generate oxygen inside the gel beads is to use hydrogen peroxide, which is decomposed to oxygen and water by catalase or inorganic catalysts. G. oxydans cells contain much catalase so no extra catalyst need be added⁶. In these two methods, the mass transfer resistance for oxygen is very small because oxygen is generated in situ.

The oxygen content of the production medium can be increased by additions of, for example, hemoglobin or emulsions of perfluorochemicals¹⁴. Emulsions of perfluorochemicals increase the amount of oxygen in the bulk liquid phase while hemoglobin, which can enter the gel, also facilitates internal oxygen transfer to some extent.

Artificial electron acceptors such as p-benzoquinone can be used in much higher concentrations than oxygen. Thereby both external and internal mass transfer is facilitated. The diffusivity of the electron acceptor should be as high as possible, thus, the molecules should be small. The kinetic constants (k_2 and K) are also of importance. The ideal electron acceptor has high solubility, high diffusivity, high k_2 and low K . The chemical p-benzoquinone meets most of these demands but perhaps even better electron acceptors could be found.

Perhaps it might be possible to find quinones that do not form quinhydrone like compounds or to decrease the tendency of quinhydrone formation by the addition of organic solvents¹⁵. It is also possible that quinones with a higher solubility than p-benzoquinone could be found.

It is interesting to see how increased concentrations of substrate affect the overall effectiveness factor if the formation of quinhydrone and the limited solubility of p-benzoquinone is neglected. The results of such calculations are shown in figure 10. Apparently, much can be gained if the substrate concentration can be increased. If an ideal electron acceptor could be found, the effectiveness factor could approach unity even with rather big particles and high cell densities.

In all the calculations, the overall effectiveness factor has been determined. However, for practical considerations, it is more important to know the formation rate of the desired product. The maximal dihydroxyacetone production rate with p-benzoquinone as electron acceptor is about 5 times that obtained with oxygen. This means that if the same overall effectiveness factor is achieved in two identical reactors operated with either p-benzoquinone or oxygen as the electron acceptor, the reactor with p-benzoquinone will produce 5 times the amount of product obtained with the reactor using oxygen. This large difference in maximal reaction rate and the high solubility of p-benzoquinone compared to oxygen make p-benzoquinone a more efficient electron acceptor than oxygen for the production of dihydroxyacetone by immobilized G. oxydans cells. There are also factors favouring the use

of oxygen. The K value is lower and the diffusivity is higher for oxygen than for p-benzoquinone but these factors are less important than the factors favouring the use of p-benzoquinone.

From this study, it is evident that when beads with about 1 mm radii and high cell densities are used, the internal mass transfer is the main rate limiting process. External mass transfer can become important if the stirring is not as efficient as in this study and if smaller beads are used. However, the efforts to increase oxygen supply to cells immobilized in gel beads ought to focus on the problems concerning internal mass transport. If the activity of the immobilized cells are to approach the activity of free cells, it is necessary that the internal mass transfer resistance be overcome. The most promising methods are in situ oxygen generation and the use of artificial electron acceptors.

ACKNOWLEDGEMENTS

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LIST OF SYMBOLS

c_{bs}	bulk substrate concentration, kmol/m^3
c_{is}	internal substrate concentration, kmol/m^3
c_{os}	substrate concentration at particle surface, kmol/m^3
d_p	particle diameter, m
D	external substrate diffusivity, m^2/s
D_{is}	internal substrate diffusivity, m^2/s
k_2	kinetic constant, $\text{kmol/s}\cdot\text{kg}$
K	Michaelis-Menten constant, kmol/m^3
k_c	mass transfer coefficient, m/s
r	distance coordinate, m
r_0	particle radius, m
Re	Reynolds number
Sc	Schmidt number
T	temperature, K
v_{free}	reaction rate of free cells, $\text{kmol/m}^3\cdot\text{s}$
v_{loc}	local reaction rate, $\text{kmol/m}^3\cdot\text{s}$
v_{obs}	observed reaction rate, $\text{kmol/m}^3\cdot\text{s}$
x	dimensionless distance coordinate ($=r/r_0$)
α	Sherwood number
β	Damköhler number
μ	viscosity, $\text{kg/m}\cdot\text{s}$
η_0	overall effectiveness factor for catalyst particle
ρ	cell density, kg/m^3
(O_2)	oxygen
$(p-b)$	p-benzoquinone
calc	calculated
exp	experimental

REFERENCES

1. V. Kasche, Enzyme Microb. Technol., 5, 2 (1983).
2. H. Hiemstra, L. Dijkhuizen and W. Harder, Eur. J. Appl. Microbiol. Biotechnol., 18, 189, (1983).
3. K. Sato and K. Toda, J. Ferment. Technol., 61, 239 (1983).
4. P. Adlercreutz, O. Holst and B. Mattiasson, Appl. Microbiol. Biotechnol. (in press).
5. P. Adlercreutz and B. Mattiasson, Appl. Microbiol. Biotechnol., 20, 296 (1984).
6. O. Holst, SO. Enfors and B. Mattiasson, Eur. J. Appl. Microbiol. Biotechnol., 14, 64 (1982).
7. D.J. Fink, T.Y. Na and J.S. Schultz, Biotechnol. Bioeng., 15, 879 (1973).
8. P. Harriott, A.I.Ch.E.J., 8, 93 (1962).
9. C.N. Satterfield, Mass transfer in heterogeneous catalysis. MIT Press Cambridge, Massachusetts and London, England (1970).
10. M.v. Stackelberg, M. Pilgram and V. Toome, Zeitschr. Elektrochemie, 57, 342 (1953).
11. J.H. Perry, Chemical Engineer's Handbook, 4th Ed., Mc Graw-Hill, New York (1963).
12. D.L. Regan, M.D. Lilly and P. Dunnill, Biotechnol. Bioeng., 16, 1081 (1974).
13. P. Adlercreutz, O. Holst and B. Mattiasson, Enzyme Microb. Technol., 4, 395 (1982).
14. P. Adlercreutz and B. Mattiasson, Eur. J. Appl. Microbiol. Biotechnol., 16, 165 (1982).
15. R.E. Moser and H.G. Cassidy, J. Am. Chem. Soc., 87, 3463 (1965).

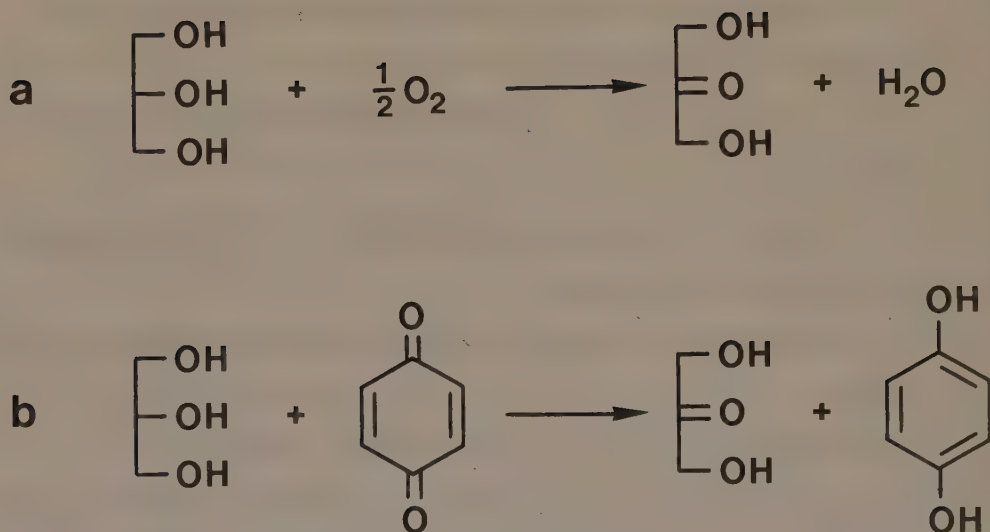


Figure 1. Formulas for the production of dihydroxyacetone from glycerol by Gluconobacter oxydans cells. In the normal reaction (a) oxygen was used as the electron acceptor but an artificial electron acceptor (p-benzoquinone) (b) can replace oxygen. Reduction of p-benzoquinone produces hydroquinone.

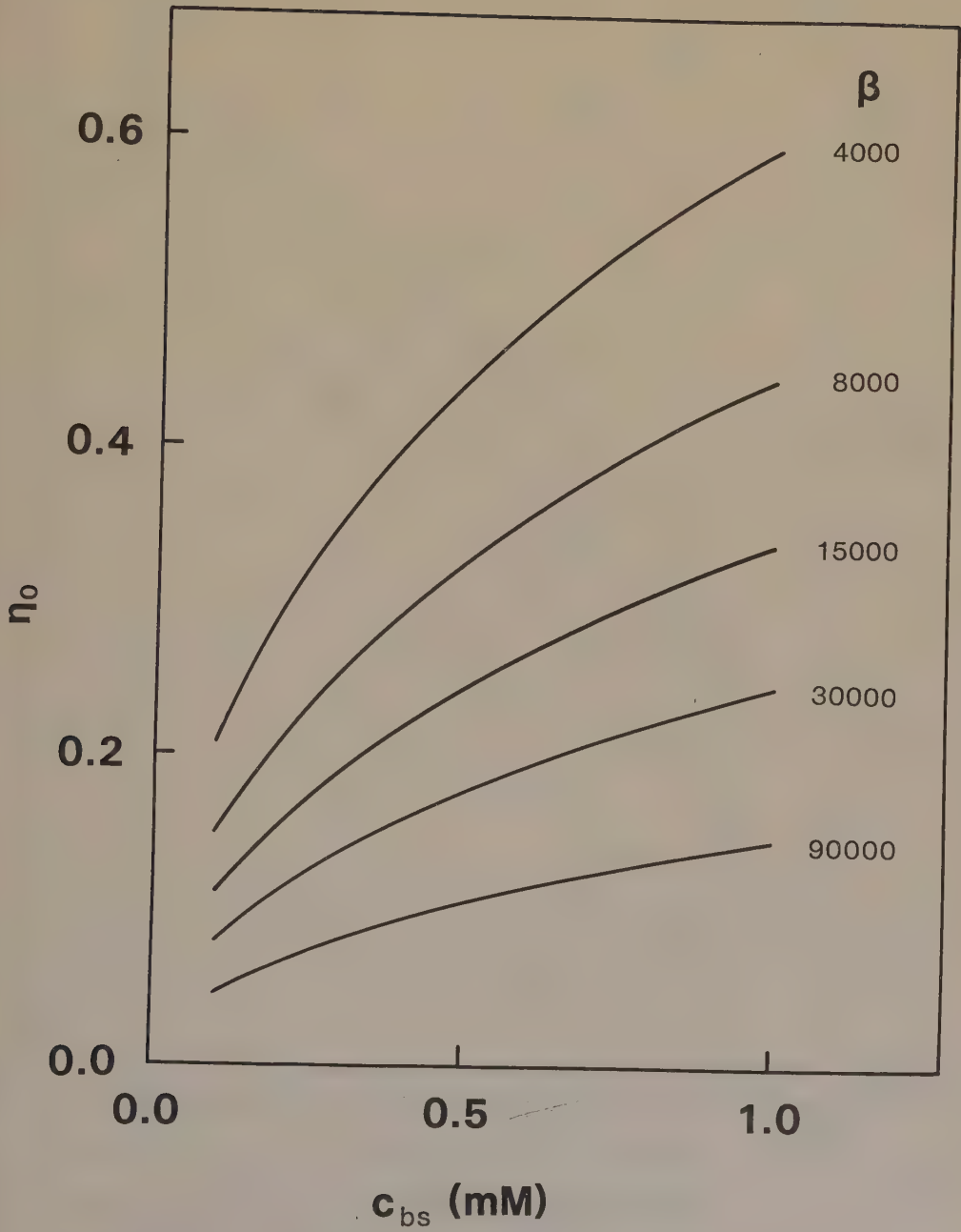


Figure 2. The overall effectiveness factor, η_0 , as a function of the bulk oxygen concentration, c_{bs} , for different β values. External mass transfer resistance was neglected ($\alpha = \infty$).

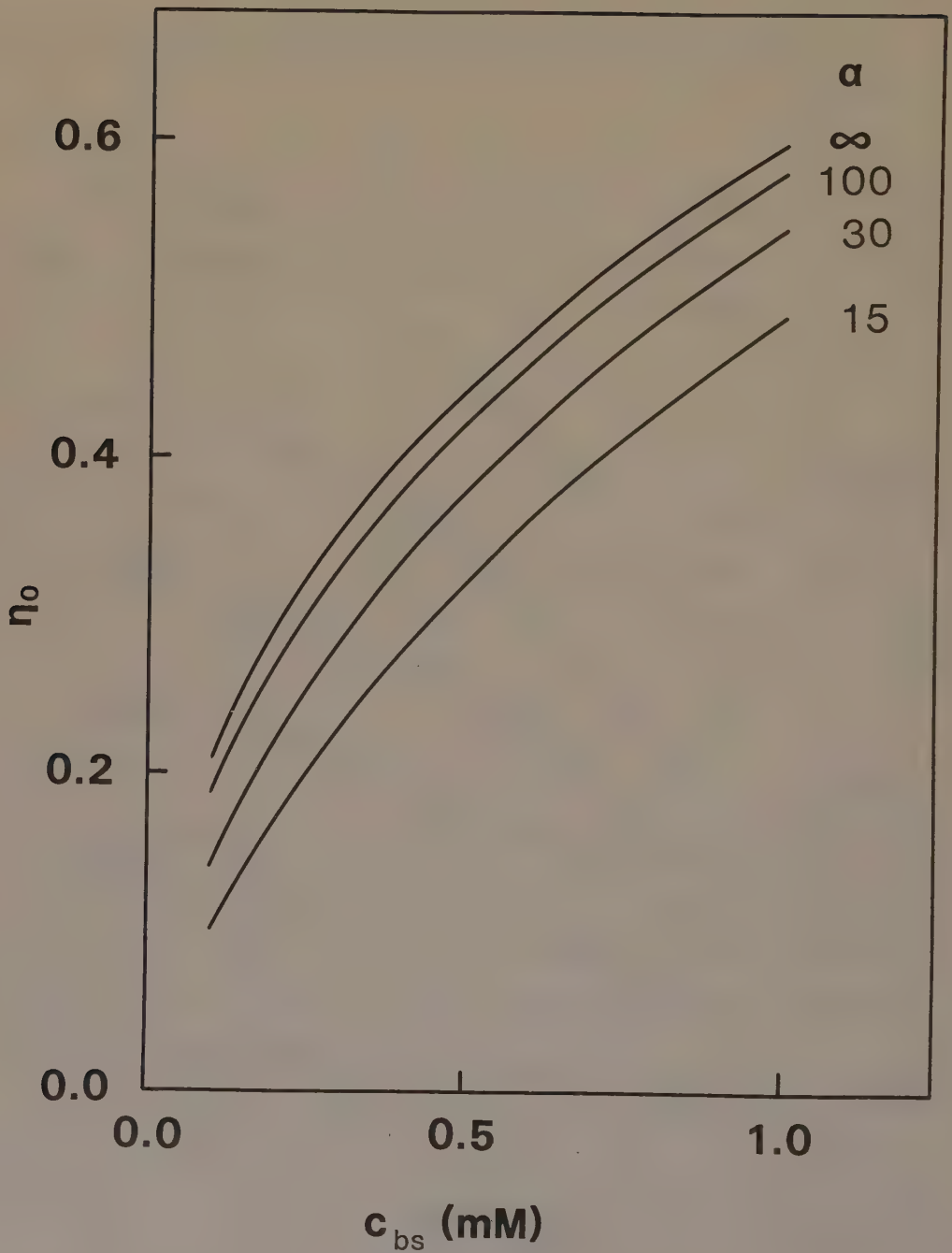


Figure 3. The overall effectiveness factor, η_0 , as a function of the bulk oxygen concentration, c_{bs} , for different α values. The β value was 4000.

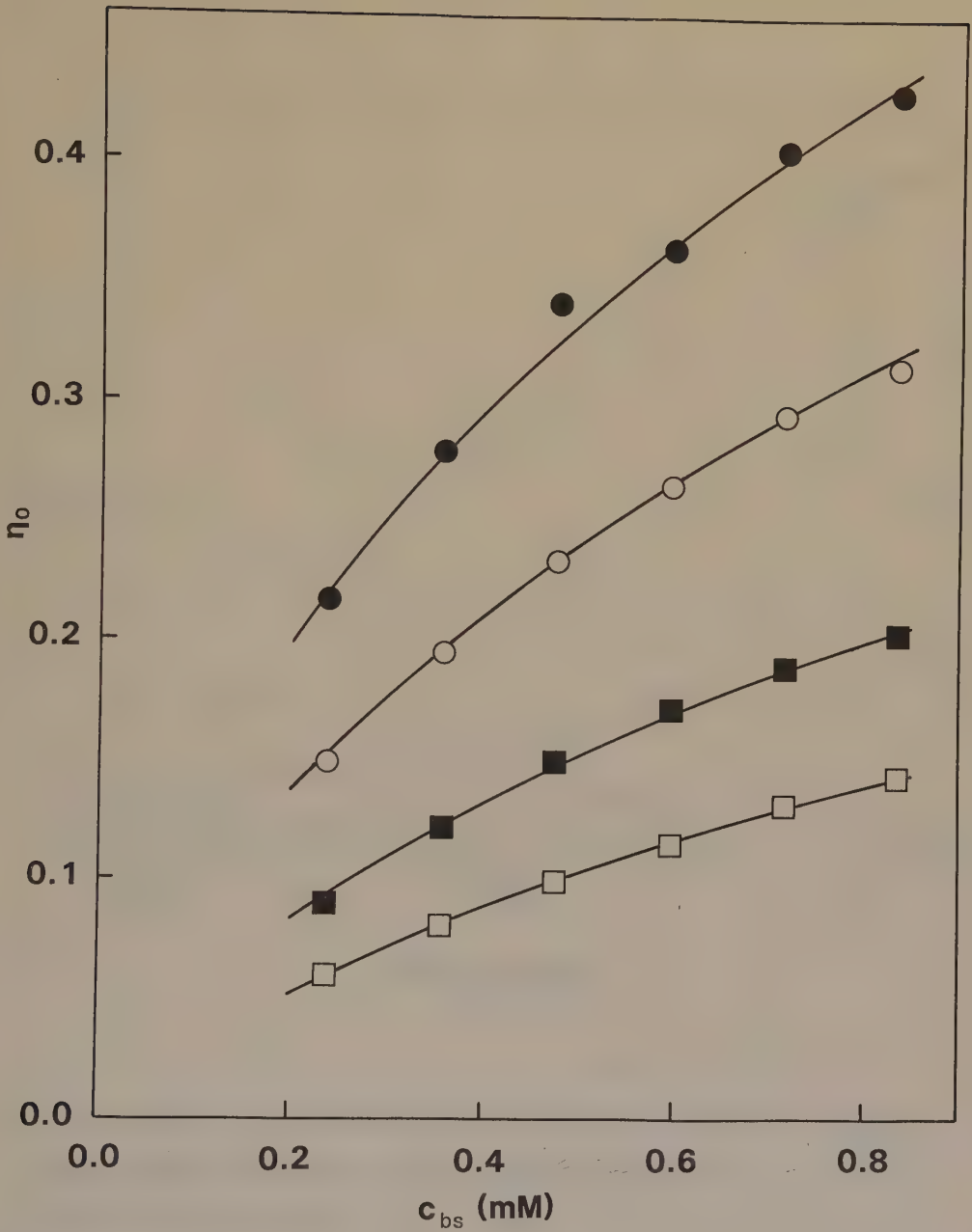


Figure 4. Comparison of theoretical calculations (solid lines) and experimental data (discrete points) for experiments with oxygen as the electron acceptor. For each immobilized preparation, the values of α and β which gave the best correlation with the experimental data were chosen. These α and β values are given in table 1. The particle radii were 1.1-1.2 mm. The following cell densities were used: 8.9 kg/m³ (●), 16.3 kg/m³ (○), 29.2 kg/m³ (■) and 48.1 kg/m³ (□).

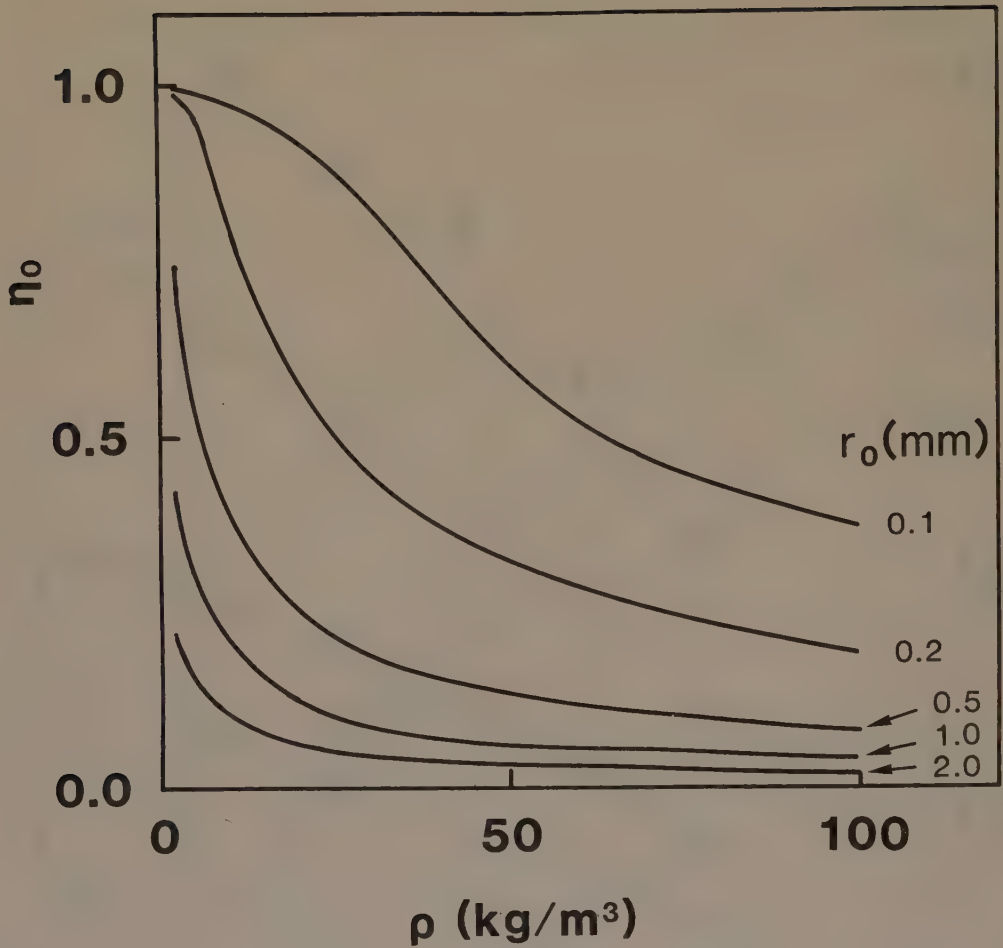


Figure 5. Theoretical calculations of the overall effectiveness factor, η_0 , as a function of the cell density for different particle radii with oxygen as the substrate. The α values were calculated according to equation (12). $D_{is}(\text{O}_2) = 2.11 \cdot 10^{-9} \text{ m}^2/\text{s}$ and $c_{bs} = 0.216 \cdot 10^{-3} \text{ kmol/m}^3$ (air saturation).

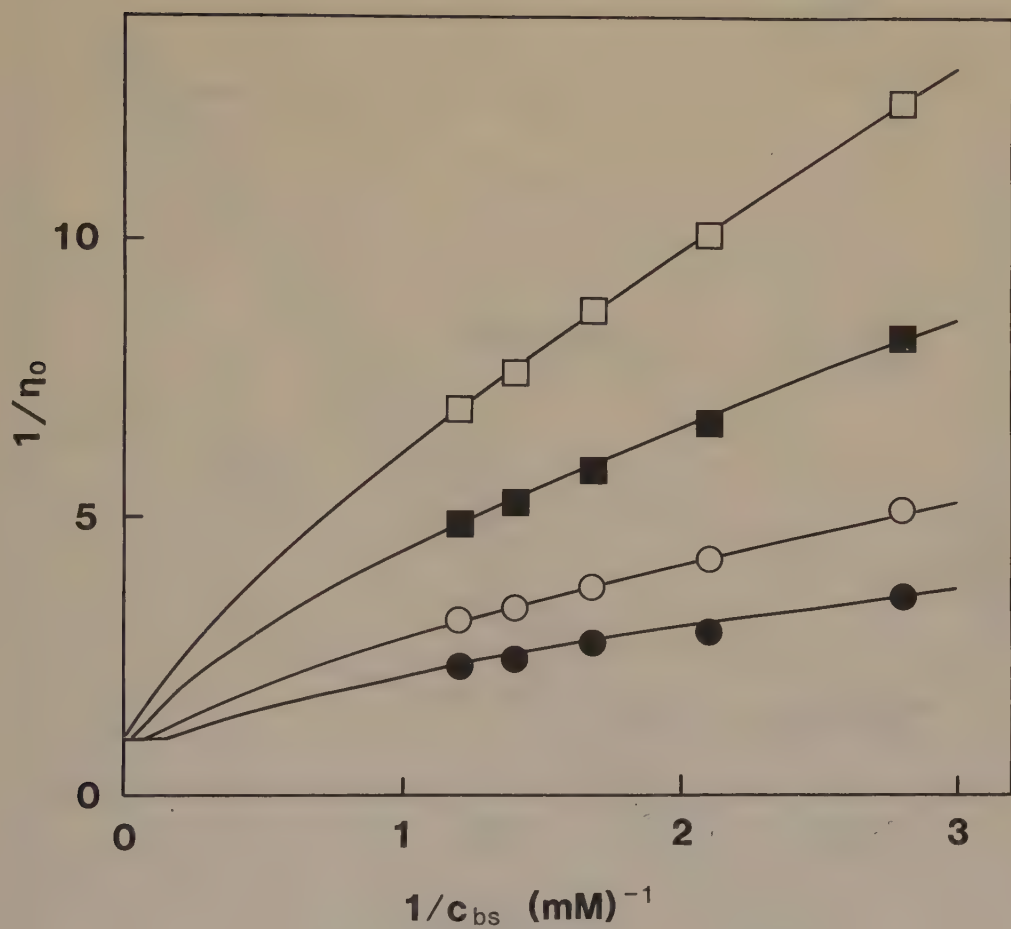


Figure 6. Double reciprocal plot. Some data from figure 4 were plotted in the double reciprocal way.

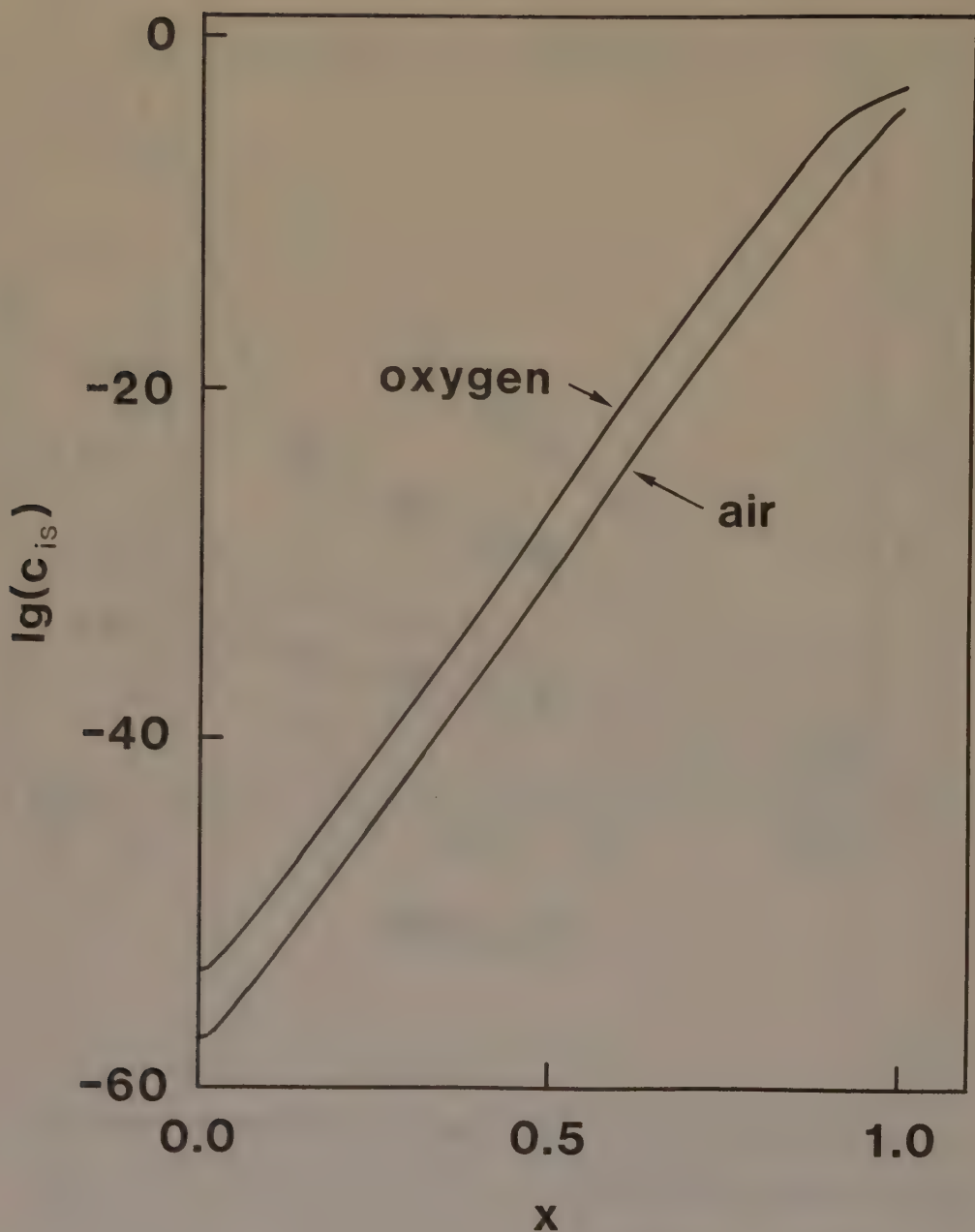


Figure 7. Concentration profiles of oxygen in gel beads. Theoretical calculation using the following parameters: $\alpha = 39$, $r_0 = 1.0 \cdot 10^{-3}$ m, $\rho = 30$ kg/m³, $D_{is} = 2.11 \cdot 10^{-9}$ m²/s, $c_{bs} = 0.216 \cdot 10^{-3}$ kmol/m³ (air saturation) or $c_{bs} = 1.034 \cdot 10^{-3}$ kmol/m³ (oxygen saturation).

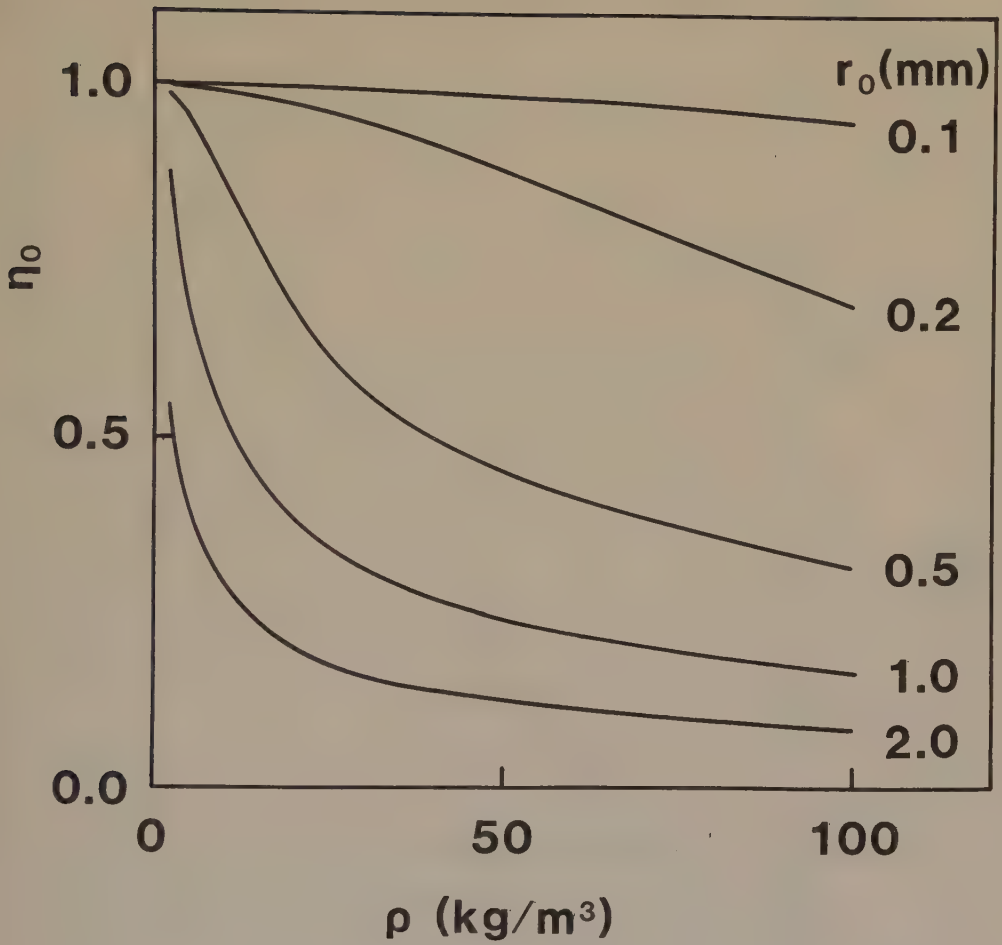


Figure 8. Theoretical calculations of the overall effectiveness factor, η_0 , as a function of the cell density for different particle radii with p-benzoquinone as the substrate. The α values were calculated according to equation (15). $D_{iS}(\text{p-b}) = 7.2 \cdot 10^{-10} \text{ m}^2/\text{s}$ and $c_{bS} = 4.0 \cdot 10^{-2} \text{ kmol/m}^3$.

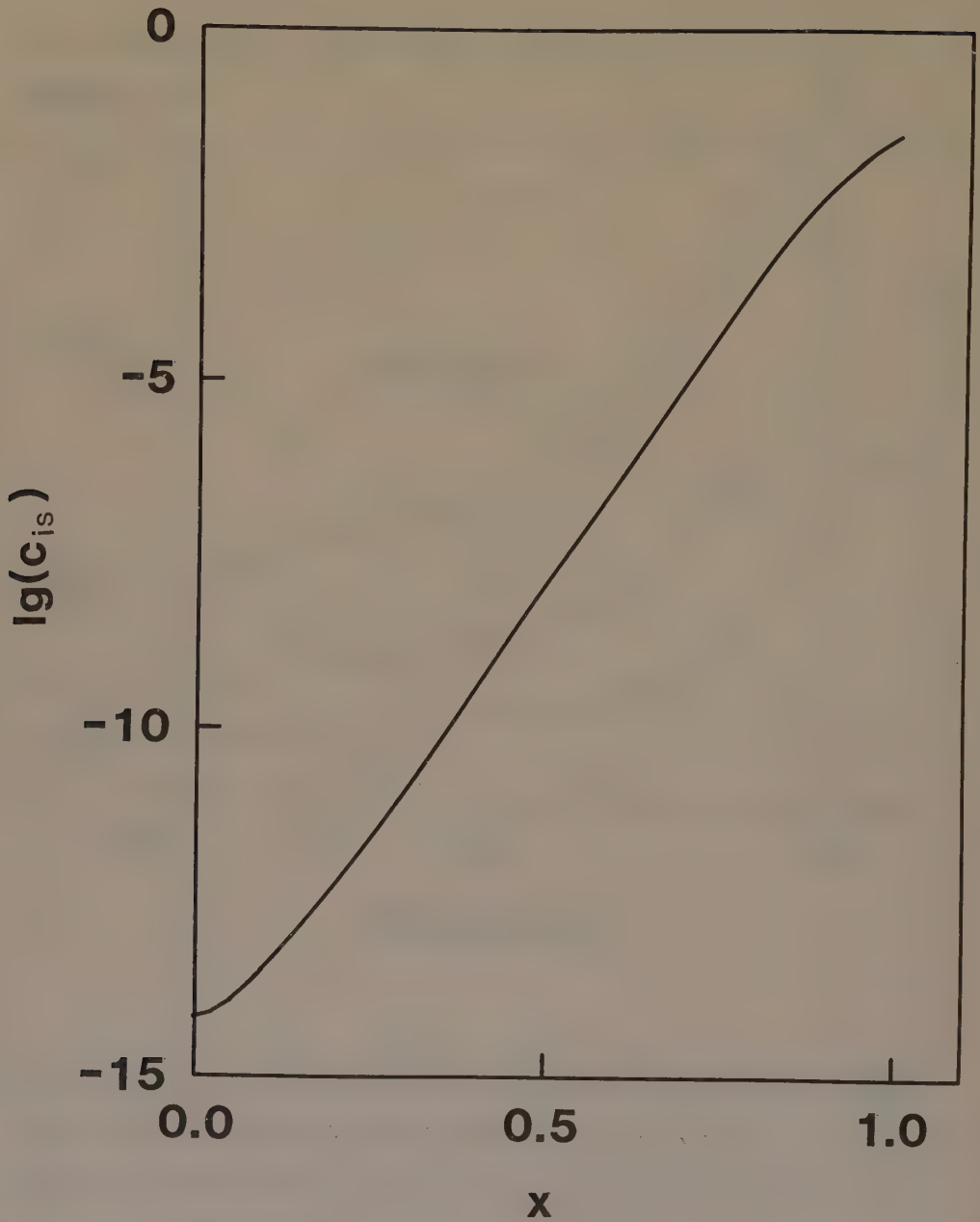


Figure 9. Concentration profile of p-benzoquinone in a gel bead. Theoretical calculations using the following parameters: $\alpha = 65$, $r_0 = 1.0 \cdot 10^{-3}$ m, $\rho = 30$ kg/m³, $D_{is} = 7.2 \cdot 10^{-10}$ m²/s and $c_{hs} = 4.0 \cdot 10^{-2}$ kmol/m³.

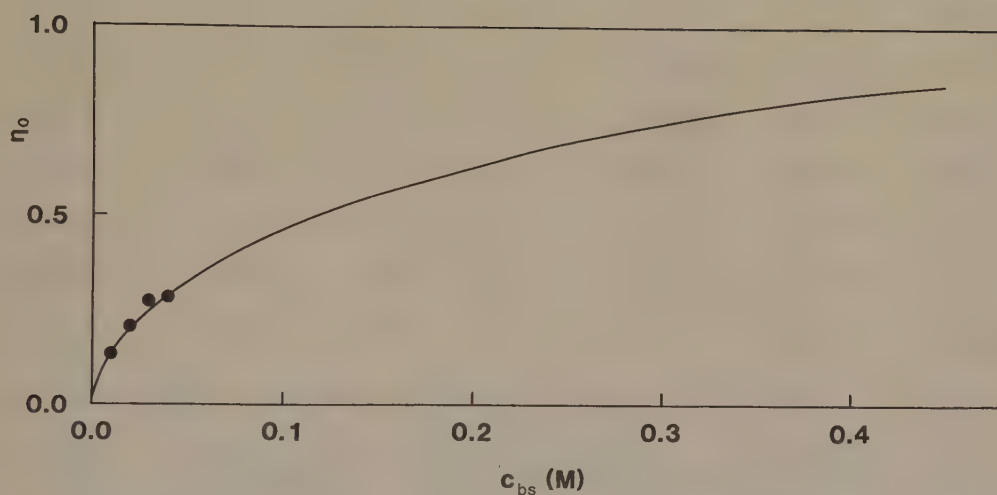


Figure 10. The overall effectiveness factor as a function of the bulk concentration of p-benzoquinone. The results of theoretical calculations neglecting the limited solubility of p-benzoquinone (about 0.1 M in the buffer used) and the formation of quinhydrone are shown as a solid curve, while experimental values are shown as discrete points. The curve was calculated using the following parameters: $\alpha = 70$, $r_0 = 1.08 \cdot 10^{-3} \text{ m}$, $\rho = 31.5 \text{ kg/m}^3$, and $D_{js} = 7.2 \cdot 10^{-10} \text{ m}^2/\text{s}$.

$r_0 \cdot 10^3$	ρ	$\eta_{o,exp}$ 1)	α_{exp} 2)	β_{exp} 2)	$D_{is} \cdot 10^9$ 3)	$\eta_{o,calc}$ 4)
1.09	8.9	0.333	52	5900	2.15	0.321
1.10	16.3	0.240	43	10200	2.33	0.227
1.14	29.2	0.152	56	24600	1.84	0.155
1.18	48.1	0.103	45	41100	1.97	0.108
1.14	29.2	0.153	36	20000	2.27	0.155
1.32	26.3	0.134	42	26700	2.07	0.143
1.64	25.4	0.117	55	37000	2.24	0.118
1.90	25.3	0.102	78	54300	2.03	0.102

Table 1. Data for the oxidation of glycerol to dihydroxyacetone by immobilized Gluconobacter oxydans cells with oxygen as the electron acceptor.

1) Experimentally determined overall effectiveness factors obtained with a bulk substrate concentration of $0.5 \cdot 10^{-3}$ kmol/m³.

2) The α and β values which gave the best correlation with the experimental data in the bulk substrate concentration range $(0.2-1.0) \cdot 10^{-3}$ kmol/m³.

3) Internal diffusivity of oxygen calculated from β_{exp} values using equation (9).

4) Calculated overall effectiveness factors. Data used: r_0 , ρ , $k_2 = 1.036 \cdot 10^{-5}$ kmol O₂/s·kg, $K = 8.6 \cdot 10^{-6}$ kmol/m³, $D_{is} = 2.11 \cdot 10^{-9}$ m²/s and $c_{bS} = 0.5 \cdot 10^{-3}$ kmol/m³. The α values were calculated using equation (12).

$r_0 \cdot 10^3$	ρ	$c_{bs} \cdot 10^3$	$\eta_{o,exp}$ 1)	β_{exp} 2)	$D_{is} \cdot 10^{10}$ 3)	$\eta_{o,calc}$ 4)
1.05	8.6	20	0.410	372	7.71	0.399
1.05	8.6	40	0.569	360	7.97	0.549
1.06	16.6	20	0.279	811	6.96	0.286
1.06	16.6	40	0.401	787	7.17	0.405
1.08	31.5	20	0.209	1400	8.05	0.198
1.08	31.5	40	0.282	1610	6.99	0.288
1.13	53.7	20	0.130	3240	6.42	0.141
1.13	53.7	40	0.204	3010	6.91	0.209
1.27	29.1	20	0.177	1990	7.17	0.178
1.27	29.1	40	0.263	1920	7.42	0.259
1.53	30.2	20	0.129	3640	5.92	0.146
1.53	30.2	40	0.224	2760	7.80	0.213
1.89	29.6	20	0.119	4440	7.27	0.120
1.89	29.6	40	0.181	4260	7.57	0.176

Table 2. Data for the oxidation of glycerol to dihydroxyacetone by immobilized Gluconobacter oxydans cells with p-benzoquinone as the electron acceptor.

1) Experimentally determined overall effectiveness factors.

2) The β values obtained from the experimental data.

3) Internal diffusivity of p-benzoquinone calculated from β_{exp} values using equation (9).

4) Calculated overall effectiveness factors. Data used: r_0 , ρ , c_{bs} , $k_2 = 1.069 \cdot 10^{-4}$ kmol p-b/s.kg, $K = 3.5 \cdot 10^{-3}$ kmol/m³, and $D_{is} = 7.2 \cdot 10^{-10}$ m²/s.

The α values were calculated using equation (15).

